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13. ABSTRACT ***(Maximum 200 Words)***

The overall goals of the project were to investigate the consequences of mutations in BRCA1 and BRCA2 in transcription-coupled repair (TCR) of oxidative damage and ultraviolet light induced damage in mouse and human systems. Since others (Gowan et al, 1998) have focused on mouse cell lines, we have focused on human cell lines. We examined the removal of UV light induced cyclobutane pyrimidine dimers (CPDs) from each strand of the DHFR gene in a BRCA1 mutant human cell line HCC1937. We find no evidence for a defect in TCR. We find no evidence for a defect in TCR in a BRCA2 deficient human cell line, Capan-1. Hence, defects in BRCA1 and BRCA2 do not appear to influence transcription-coupled repair of UV damage. The nucleotide excision repair pathway removes UV light-induced damage and bulky adducts formed in DNA by certain carcinogens. Others (Gowan et al, 1998) have reported that mouse embryo fibroblasts deficient in BRCA1 and BRCA2 are deficient in TCR of oxidative damage that includes the lesion thymine glycol (Tg). Many forms of oxidative damage including thymine glycol are removed by base excision repair that is a repair pathway distinct from nucleotide excision repair. Transcription-coupled repair of Tg has been measured using an antibody specific to Tg in an immunoprecipitation reaction. We were not successful in obtaining reproducible results using this procedure and hence, were unable to measure the consequences of BRCA1 and BRCA2 in TCR of Tg.

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breast cancer, TCR, BRCA1, BRCA2

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INTRODUCTION:

If DNA damage is left unrepaired in can result in the accumulation of mutations. Genetic instability and genome-wide accumulation of mutations drive tumorigenesis. Cells possess a variety of DNA repair pathways that remove DNA damage, reduce mutations and help protect humans from cancer (Friedberg, Walker et al. 1995). Two important repair pathways are nucleotide excision repair (NER) and base excision repair (BER). Transcription-coupled repair (TCR) has been clearly shown to be a sub-pathway of NER. TCR is often observed as more rapid or more efficient removal of bulky adducts from the transcribed strands of active genes compared with the nontranscribed strands (Scicchitano and Mellon 1997; Mellon 2002). TCR has also been proposed to be a sub-pathway of BER of oxidative damage (Leadon and Lawrence 1992; Leadon and Cooper 1993; Cooper, Nouspikel et al. 1997). Oxidative damage and bulky carcinogen-induced damage could play roles in the etiology of breast cancer. Inherited genetic defects in BRCA1 and BRCA2 clearly increase the risk of the development of breast cancer. Hence, the goal of the proposed work was to test the hypothesis that genetic defects in BRCA1 and/or BRCA2 reduce or abolish TCR of either oxidative damage or bulky damage to determine whether defects in TCR play roles in the etiology of breast cancer.

BODY:

(A) One of the goals was to measure the removal of cyclobutane pyrimidine dimers (CPDs) from each strand of the DHFR gene in BRCA1 or BRCA2 mutant cell lines. We encountered technical difficulty with the strand-specific probes used to study the human DHFR gene. We focused on human cells because the Leadon laboratory (Gowen, Avrutskaya et al. 1998) had reported on mouse embryo fibroblasts and found no evidence for a defect in TCR of UV damage. We had provided them with the mouse probes to study the mouse DHFR gene (Gowen, Avrutskaya et al. 1998). We also had problems growing sufficient quantities of some of the mutant cell lines required to perform the TCR assay. Eventually we were successful and found that mutations in BRCA1 and BRCA2 in human cells had no effect on TCR of UV damage. Our results confirm those previously reported for mouse cells (Gowen, Avrutskaya et al. 1998). Interestingly a recent study has found that BRCA1 specifically enhances the global genomic repair sub-pathway of NER (Hartman and Ford 2002). We too observed a reduction in the repair of CPDs from nontranscribed DNA in the BRCA1 mutant cell line.

(B) The other goal was to measure the removal of oxidative damage from each strand of the DHFR gene to determine whether mutations in BRCA1 and or BRCA2 influence TCR of oxidative damage. Reduced TCR of oxidative damage has been found in BRCA1 mutant mouse embryo fibroblast cell lines (Gowen, Avrutskaya et al. 1998). Their approach was to measure the removal of oxidative damage from each strand of an active gene using an antibody to thymine glycol (Tg) in an immunoprecipitation reaction. We obtained the Tg specific antibody from the Leadon laboratory. We were successful in using it

to measure global genome repair of Tg (see appendix and (Alanazi, Leadon et al. 2002). However, we were unsuccessful using the antibody to measure TCR of Tg either in bacterial cells or from mammalian cells. The Alanazi et al paper is included in the appendix to illustrate that the Tg antibody is appropriate for global genome repair studies but it is clearly problematic for TCR studies. This item in the appendix was not part of this project but similar experiments were attempted with mammalian cell lines. Other laboratories have had similar difficulties (Philip Hanawalt, Stanford University, personal communication). Hence, there is concern about the role of BRCA1 and or BRCA2 in TCR of Tg. However, Abbott et al, (Abbott, Thompson et al. 1999) have confirmed an association between BRCA1 and TCR of oxidative damage using an antibody to bromo-uracil (BU). However the BU antibody lacks any specificity to follow a specific lesion or a specific repair pathway which is why we did not pursue using it. Hence, it remains unclear whether mutations in BRCA1 confer any defect in a base excision repair pathway.

(C) While we were working on both the UV experiments and on the oxidative damage experiments we decided to establish methods where we could construct mutant human cell lines to determine the consequences of alterations in BRCA1 or BRCA 2 on DNA repair. Since my laboratory has not been involved in constructing mutant mammalian cell lines in the past, I chose to establish control cell lines using the XPA gene which is an essential gene for NER and TCR of UV damage. With this approach we could construct mutant cell lines and measure the consequences for repair and cell survival using established methods in my laboratory with known predictable outcomes. We chose a hormone inducible expression vector that would allow for basal low levels of expression and increased induced levels of expression. We successfully used this approach to construct XPA wild type and mutant cell lines and measured their consequences for NER, TCR and cell survival (appendix and (Mellon, Hock et al. 2002)). We did not extend these studies to BRCA1 or BRCA2 because it became clear that we were seeing no effect of either gene on TCR of UV damage (A) and we could not measure its effect on oxidative damage (B).

KEY RESEARCH ACCOMPLISHMENTS:

(1) We have found that defects in either BRCA1 or BRCA2 do not reduce or abolish transcription-coupled repair of UV light induced DNA damage in human cells. Hence, we find no evidence that defective TCR of bulky adducts is involved in the etiology of breast cancer associated with mutations in either BRCA1 or BRCA2.

(2) We have found that measuring transcription-coupled repair of thymine glycol with an antibody to Tg in an immunoprecipitation assay is unreliable. It is unclear whether a defect in TCR of Tg is associated with BRCA1 and/or BRCA2 associated breast cancer.

REPORTABLE OUTCOMES:

1) Mellon, I., Hock, T., Reid R., Porter, P.C., and States, J.C.; Polymorphisms in the human xeroderma pigmentosum group A gene and their impact on cell survival and nucleotide excision repair, (2002) *DNA Repair*,**1**, 531-546.

CONCLUSIONS:

(1) BRCA1 and BRCA2 are not involved in transcription-coupled repair of UV damage.
(2) It is unclear whether either BRCA1 or BRCA is involved in TCR of thymine glycol.

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APPENDICES:

1) Mellon, I., Hock, T., Reid R., Porter, P.C., and States, J.C.; Polymorphisms in the human xeroderma pigmentosum group A gene and their impact on cell survival and nucleotide excision repair, (2002) *DNA Repair*, **1**, 531-546.

2) Alanazi, M., Leadon, S. A. and Mellon, I. (2002) Global genome removal of thymine glycol in *Escherichia coli* requires endonuclease III but the persistence of processed repair intermediates rather than thymine glycol correlates with cellular sensitivity to high doses of hydrogen peroxide, (2002) *Nucleic Acids Research*, In Press.

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Polymorphisms in the human xeroderma pigmentosum group A gene and their impact on cell survival and nucleotide excision repair

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Abstract

Polymorphisms in DNA repair genes may contribute to defects in DNA repair and increased susceptibility to cancer. The xeroderma pigmentosum group A (*XPA*) gene is required for nucleotide excision repair (NER) and mutations in *XPA* highly predispose humans to skin cancer. We examined DNA samples from 189 individuals for polymorphisms in the *XPA* gene. First, SSCP analysis was used to examine each of the six exons and their intron boundaries. One frequent single nucleotide polymorphism (SNP) in the untranslated region of exon 1 and two rare SNPs which produce the changes Arg228Gln and Val234Leu in the coding region of exon 6 were identified. Quite surprisingly, no sequence variants were found within the coding regions or the adjacent intron boundaries of exons 1–5. Ecdysone-inducible expression vectors containing wild type *XPA* cDNA or cDNAs representing the two polymorphisms that we identified in exon 6 were created and independently introduced into the *XPA* deficient cell line XP12RO-SV. Transcription-coupled repair (TCR), global genome repair (GGR) and cell survival following UV irradiation were studied in each cell line in the absence or presence of the ecdysone hormone analog, ponasterone A. No substantial difference in repair or cell survival was found in cells complemented with wild type or polymorphic alleles of *XPA*. A 10-fold increase in the expression of XPA by addition of ponasterone A resulted in faster removal of 6–4 photoproducts from the total genomes of cells complemented with wild type or polymorphic alleles of *XPA* but had no significant impact on TCR or global genome repair of cyclobutane pyrimidine dimers (CPDs). Since our SSCP analysis failed to detect significant numbers of polymorphisms we directly sequenced exons 4–6 in a subset of our samples. One additional rare SNP, which produces the change Leu252Val was found in exon 6 and four rare SNPs and one rare single nucleotide deletion were found in intron 4. Hence, the *XPA* gene appears to be a cold spot for genetic variation and rare polymorphisms in the coding region of the gene do not reduce NER or cell survival after UV irradiation.

Keywords: Genetic polymorphisms; DNA repair; Transcription-coupled repair; Global genome repair; Xeroderma pigmentosum; *XPA* gene

1. Introduction

Nucleotide excision repair (NER) is the major pathway in humans for the removal of CPDs and 6–4 photoproducts produced by ultraviolet light [1]. NER also removes a wide variety of bulky lesions formed by chemical agents. It is a complex multi-step process

Abbreviations: CPD, cyclobutane pyrimidine dimer; TCR, transcription-coupled repair; SNP, single nucleotide polymorphism; SSCP, single strand conformation polymorphism; XP, xeroderma pigmentosum

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that requires the participation of at least 30 proteins [2,3] and involves damage recognition, unwinding of the DNA helix near the lesion, dual incisions (one on each side of the lesion), removal of a stretch of DNA containing the lesion, re-synthesis of DNA to replace the damaged DNA and ligation. There are two subpathways in NER [4]: one is termed TCR which selectively removes lesions from the transcribed strands of expressed genes and the other is global genome repair (GGR) which removes lesions from the remainder of the genome. CPDs and 6–4 photoproducts are substrates for both subpathways of NER [5] but 6–4 photoproducts are removed more rapidly than CPDs from most of the genome [6,7].

XP is a rare genetic disease with an autosomal recessive mode of inheritance [8–11]. Patients are extremely sensitive to sunlight and display freckling and other pigmentation abnormalities in sun-exposed areas of the skin. A hallmark of the disease is a 1000-fold increase in skin cancer frequency [12]. In addition, a subset of patients develop neurological abnormalities [13]. XP is genetically complex with eight complementation groups; seven are defective in NER (XPA to XPG) and one is defective in translesion DNA synthesis at UV photoproducts (XPV) [14]. The high frequency of skin cancer associated with XP is caused by the reduced ability to process sunlight-induced UV photoproducts, which results in an increased mutational load and neoplastic transformation.

Of the seven XP gene products involved in NER, three are involved in damage recognition, two are involved in unwinding the DNA helix and two are nucleases. The identity of XP proteins involved in the damage recognition step has been controversial [3]. The candidates are XPA–replication protein A (RPA) complex, XPC–hHR23 complex and p48–p127 complex (p48 is mutated in XPE cells [15,16]). Recently XPC–hHR23B was shown to be the first NER protein to bind 6–4 photoproducts during GGR in vivo [17] supporting previous observations that XPC–hHR23B is a key player in the initial step of damage recognition [18,19]. XPA may not be involved in the first step of damage recognition but it interacts with several other NER proteins including RPA [20], ERCC1–XPF [21–23] and TFIIH [24,25] and likely plays a crucial role in the formation of the pre-incision complex [17].

The inheritance of two mutant alleles of an XP gene can highly predispose humans [9,12] and mice [26–30] to cancer. While XP genes play a crucial role in protecting against sunlight-induced skin cancers, their protective role against non-cutaneous tumors in humans is difficult to evaluate but XP patients below the age of 20 exhibit a 10–20-fold increase in internal cancers [31]. In contrast, XP mutations in mice clearly confer an increase in internal cancers and produce elevated frequencies of benzo[a]pyrene-induced lung tumors [32], 2-acetylaminofluorene-induced liver and lung tumors and spontaneous testicular tumors [33]. It is controversial as to whether human carriers of a single mutant allele are in fact predisposed to cancer but there is evidence that family members of XP patients may be at greater risk for developing skin cancer [34]. There are also reports of diminished repair and increased chromosomal instability in XP carriers [35–39], but others find no evidence for diminished repair [40]. In contrast to human heterozygotes, XPC+/− mice are clearly predisposed to skin cancer [28]. The differences between rodent and human studies could be related to the rarity of the disease in humans and the inability to experiment on human subjects.

Polymorphisms in XP genes could contribute to defects in NER in the general population and increase susceptibility for skin and non-cutaneous cancers [41,42]. DNA repair capabilities vary among individuals and in at least some individuals, these variations may have a genetic basis [43]. Polymorphisms in several XP genes have been identified [42,44–51]. Polymorphisms in the *XPD* gene confer a decrease in the repair of X-ray-induced [48] and UV-induced damage [52] and have been associated with basal cell carcinoma [53,54] and possibly associated with glioma [55]. However, the functional or biological significance of most XP gene polymorphisms is largely unknown.

In the present study, we examined 189 human samples for polymorphisms in *XPA*. We focused on *XPA* because mutations in this gene confer the most severe symptoms of XP. Most XPA patients are highly predisposed to skin cancer and their cells are often completely deficient in both GGR and TCR of UV damage and are extremely sensitive to killing by UV irradiation. Therefore, polymorphisms in this NER gene may have a more measurable impact on function.

Our results suggest that polymorphic variants in XPA protein coding regions are exceedingly rare. Only a single common variant was observed in non-coding regions. We find that two of the coding variants do not decrease cell survival or repair after UV exposure.

2. Materials and methods

2.1. SSCP analysis

Human DNA samples provided by the Molecular Diagnostics Laboratory in the Department of Pathology and Laboratory Medicine at the University of Kentucky were studied. This was a largely Caucasian population and contained approximately equal numbers of males and females. Primers were used to amplify each of the coding regions of the six exons, adjacent intron boundaries for each exon, a portion of the 5′ untranslated region of exon 1, and a portion of the 3′ untranslated region of exon 6 (Table 1). The PCR reactions for exons 2–5 were performed in a 10 μl reaction volume containing 1× buffer (Applied Biosystems, Foster City, CA), 10 mM Tris–HCl (8.8), 50 mM KCl, 200 μM each dATP, dTTP, and dGTP, 22.5 μM dCTP, 1.5 mM MgCl, 0.4 μM each primer, 1 μC α-[^{32}P] dCTP, 0.5 units of *Taq* DNA polymerase (Applied Biosystems) and 50 ng of genomic DNA. The samples were initially denatured at 94 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 30 s, primer annealing at 58 °C for 30 s, primer extension at 72 °C for 30 s, and finally an extension at 72 °C for 4 min. The PCR conditions for the remaining exons had the following modifications: exon 1B, Stratagene buffer 9 (Stratagene PCR optimization kit, Stratagene, La Jolla, CA) plus 5% formamide, primer annealing at 55 °C for 30 s; exon 1D, Stratagene buffer 9 plus 0.5% Perfect Match adjunct, primer annealing at 60 °C for 30 s; exon 6A, Stratagene buffer 8, primer annealing at 55 °C for 30 s; and exon 6B, Stratagene buffer 10, primer annealing at 55 °C for 30 s. After amplification, samples were denatured for 5 min, chilled on ice and electrophoresed under non-denaturing conditions in 0.5× MDE (BioWhittaker, Rockland, ME) gels with or without glycerol in the cold or at room temperature. Gels were dried and exposed to X-ray film. Bands of interest were excised from the gel, re-amplified, passed through a QIAquick column (Qiagen, Valencia, CA) and sequenced with the same primers used for amplification and an ABI PRISM Model DNA 377 Sequencer (Applied Biosystems). When a polymorphism was identified, the region was amplified from the patient's genomic DNA as described above, passed through a QIAquick column and re-sequenced.

2.2. DNA sequencing analysis

A subset of the 189 DNA samples used in the SSCP analysis were sequenced in regions containing exons 4–6. Primers were designed and used to amplify the coding regions and adjacent sequences of exons 4–6 (Table 1). The PCR reactions for the exons were performed using 19 μl PCR SuperMix High Fidelity (22 U/ml *Taq* and *Pyrococus* species GB-D polymerase mix, 66 mM Tris–SO_4 (pH 9.1), 19.8 mM $(NH_4)_2SO_4$, 2.2 mM $MgSO_4$, 220 μM dATP, dTTP, dGTP, and dCTP (Invitrogen, Carlsbad, CA), 15 ng genomic DNA and 10 pmol of each amplification oligo in a total volume of 21 μl. For exon 4 amplification, the reaction mixtures were initially denatured at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, primer annealing at 56 °C for 30 s, primer extension at 72 °C for 2.5 min, and finally an extension at 72 °C for 10 min. The PCR conditions for the remaining exons had the following modifications: exon 5 primer annealing temperature was 49.7 °C and exon 6 primer annealing temperature was 57.0 °C. After amplification, the reactions were cleaned-up using Qiagen PCR clean up kits. PCR products were quantified using PicoGreen dsDNA Quantitation Kit (Molecular Probes, Eugene, OR). The PCR products served as templates for use with a 0.5× Big Dye Terminator Fluorescent Sequencer (Applied Biosystems,). The sequencing reactions contained 4 μl Big Dye Terminator Sequencing Reaction Mix, 2 μl 5× sequencing buffer, 3.2 pmol of sequencing oligo, 3–10 ng of PCR product template, and were diluted to 20 μl with sterile water. The cycle sequencing conditions were 25 cycles of denaturation at 96.0 °C for 10 s, primer annealing at 50 °C for 5 s and primer extension at 60.0 °C for 4.0 min. The sequencing reactions were read on an ABI Prizm 310 Genetic Analyzer. The resulting sequencing traces were analyzed using DNA Star software (DNASTAR Inc. Madison, WI) with manual confirmation.

Table 1
Primers used in this study

Primer name	Sequence	Size of amplified fragment[a]	Position of amplified fragment
SSCP analysis[b]			
Exon 1B	F-TCA CTC AGA AAG GCC GCT GG	26939–26576	5′ UTR + exon 1 coding sequence + splice region
	R-TCG GCT TGC ACG AGC CAG TC	(364 bp)	
Exon 1D	F-CAG GCC TGA CTC CAA AGC CT R-AGC TCC ACG CAC GCG CAC	27147–26851 (297 bp)	5′ UTR
Exon 2	F-AAG GTA ACA TAC AGG CTT ACC R-TTA TTT AGC ATC ACT TTG CAT AG	23346–23094 (253 bp)	Exon 2 + splice regions
Exon 3	F-GGC ATT GCA TAC ATG CTG ATA R-GCC CTA AAC CTA CAC ATA AAC	19263–19008 (256 bp)	Exon 3 + splice regions
Exon 4	F-CCT AAG TTG CTG GGC TAT TTG R-AAG CCA AAC CAA TTA TGA CTA G	16867–16548 (320 bp)	Exon 4 + splice regions
Exon 5	F-TTC GCA AGT CTG AAT CAC AAC R-GGT AAA ACA CAA TCC TTC ACG	14618–14410 (209 bp)	Exon 5 + splice regions
Exon 6A	F-GGA TTC ACC TGA ATA GCA CC R-TTT TCA TAT GTC AGT TCA TGG C	5169–4975 (195 bp)	Exon 6 + splice region
Exon 6B	F-TAC TAT GTG TGG CCA TGA ACT	5007–4760	Exon 6 + 3′ UTR and splice region
	R-ACA TTG TGC ACA CAA CCA GG	(248 bp)	
Direct sequencing analysis amplification primers			
Exon 4	F-AAG Ttg CTG GGC TAT TTG R-GTT TTT CCA CAC TCT GTA AG	16864–16525 (340 bp)	Exon 4 and flanking
Exon 5 introns	F-CAG TTT CAT AGG TTT AGA TAG A R-AAA ACA CAA TCC TTC A	14770–14413 (358 bp)	Exon 5 and flanking
Exon 6 introns	F-AGC TTG ATG GAG TTG GAT R-GGT TTC ATT CAT CTA TGA AGA TGT TGC	5191–4854 (338 bp)	Exon 6 and flanking
Sequencing primers (designed for both strands)			
Exon 4	F-CTG GGC TAT TTG CAA ACT TAG CTT A R-GTA AGC AAA AGC CAA ACC AAT TAT G	16858 16540	Intron 3 Intron 4
Exon 5	F-CAT TCT TTG GTA CCT TTG GAT TTG A R-AAT CCT TCA CGA TAT AAA ATG TGG C	14661 14420	Intron 4 Intron 5
Exon 6	F-TGG ATT TTT GGA TTC ACC TGA ATA G R-TTT TGA ATT TTG AAA AGG ACC AAT C	5178 4887	Intron 5 3′ UTR

[a] Numbers according to AL445531 (this sequence is in antisense).
[b] The same primer sets were used for amplification and sequencing.

2.3. Cell culture

The human *XPA* mutant cell line XP12RO-SV provided by Dr. Ann Ganesan (Stanford University) is an SV40-transformed cell line derived from an XPA patient homozygous for a stop codon in the *XPA* gene [56]. XP12RO-SV and the cell lines derived from XP12RO-SV constructed in this study were grown in minimal essential medium (Eagle) (containing Earle's salts, GIBCO-BRL, Rockville, MD) supplemented with 0.1 mM non-essential amino acids, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (GIBCO-BRL).

2.4. Construction of plasmids containing wild type and polymorphic cDNA alleles of XPA

A plasmid containing the complete *XPA* cDNA cloned into pET16B was provided by Dr. Kiyoji Tanaka (Osaka University) and it was used to construct the plasmids pIND-XPAwt (wild type *XPA*), pIND-R228Q (*XPA* with the Arg to Gln change at codon 228) and pIND-V234L (*XPA* with the Val to Leu change at codon 234). It was first digested with *Nde*I, then treated with Klenow fragment to fill in the *Nde*I ends, digested with *Bam*H1 and the *XPA* cDNA containing restriction fragment purified by gel electrophoresis. This fragment was cloned into the ecdysone hormone inducible expression vector pIND (InVitrogen) after pIND was first digested with *Afl*II, then treated with Klenow to fill in the *Afl*II-generated ends and subsequently digested with *Bam*H1. The resulting clone pIND-XPAwt was sequenced to confirm that this strategy resulted in the introduction of a Kozak sequence near the first codon of the *XPA* gene and that the plasmid retained the correct sequence of wild type *XPA*. To construct polymorphic *XPA* alleles identified in this study, site directed mutagenesis of pIND-XPAwt was performed using the unique site elimination (USE) kit according to the manufacturer's instructions (Amersham Pharmacia, Piscataway, NJ) with the exception that incubations with DNA polymerase were performed at 40 °C. The primer mut228, 5′gaattgcgg**caa**gcagtaagaagc3′, was used to create the R to Q change at codon 228 and the primer mut234, 5′gtaagaagcagc**ttg**tggaaaagg3′, was used to create the V to L change at codon 234. Sequencing confirmed that the resulting plasmids, pIND-R228Q and pIND-V228L, contained the correct mutations and had not acquired additional mutations elsewhere. The following primers were used for sequencing: (1) ecdysone forward primer and BGH reverse primer, both from InVitrogen, were used to sequence the 5′ and 3′ ends of the *XPA* gene insert and (2) XPAint (5′atgcgaagaatgtggga3′) and XPAintR (5′cacagtctttcagaagat3′) were used to sequence the central region of the *XPA* gene insert.

2.5. Construction of cell lines containing wild type and polymorphic alleles of XPA

The Ecdysone Inducible Mammalian Expression System (Invitrogen) was used to establish stable XP12RO-SV cell lines containing the plasmids pIND-XPAwt, pIND-R228Q or pIND-V234L. This system relies on transfection with two different vectors: pVgRXR and pIND containing wild type or variant *XPA* cDNA. The plasmid pVgRXR contains genes encoding the *Drosophila* ecdysone receptor and the mammalian *RXR* gene, which together form a functional ecdysone receptor capable of promoting transcription from ecdysone response elements. XP12RO cells (1×10^6) were transfected with 10 μg of each plasmid by electroporation using Gene Pulser (Bio-Rad, Richmond, CA). Stable transfectants were selected in growth medium containing 400 μg/ml Zeocin and 400 mg/ml G418. Four stable cell lines were established and used in this study: RXR (XP12RO-SV transfected with pVgRXR alone), XPAwt (XP12RO-SV transfected with pVgRXR and pIND-XPAwt), R228Q (SV40-SV transfected with pVgRXR and pIND-R228Q) and V234L (SV40-SV transfected with pVgRXR and pIND-V234L).

2.6. Western analysis

The relative abundance of XPA protein in the cell lines described above was determined by immunoblotting using mouse monoclonal antibodies to human XPA. Cells were lysed in buffer containing 12.5 mM Tris–HCl (6.8), 2% SDS, 2% β-mercaptoethanol, 20% glycerol, and bromophenol blue. Equal amounts of protein were boiled for 3–5 min, separated by electrophoresis in SDS/12% PAGE gels, and electroblotted to nitrocellulose membranes. The membranes were briefly allowed to dry and then incubated overnight with 1× PBS, 0.1% Tween-20, and 7% non-fat dry milk. Immunoblotting was performed by addition of mouse monoclonal antibodies to human XPA (Neomarkers, Fremont, CA 400× dilution) and incubation for an additional 1.5 h. Membranes were then washed with 1× PBS and 0.1% Tween-20 and incubated with horse radish peroxide-conjugated goat anti-mouse antibodies (5000× dilution, Sigma) for 1.5 h. After several washes in 1× PBS and 0.1% Tween-20, antibody binding to XPA was detected using enhanced chemiluminescence (Amersham). Membranes were then incubated with polyclonal antibodies to actin (1000× dilution, Sigma) and horseradish peroxidase-conjugated anti-rabbit

antibodies to detect the relative abundance of actin in each sample.

2.7. Transcription-coupled repair analysis

The removal of CPDs from the transcribed or non-transcribed strand of a 20 kb *Kpn*I restriction fragment that resides within the transcription unit of the dihydrofolate reductase (*DHFR*) gene was examined as described [57]. Cells were irradiated with UV light (10 J/m^2) and either lysed immediately or incubated for increasing periods of time to allow repair and then lysed. For experiments examining the effects of XPA induction, the ecdysone hormone analog, ponasterone A, was added to the growth medium 24 h prior to UV irradiation and for the specified periods of time following UV irradiation. DNA was isolated, treated with *Kpn*I, mock-treated or treated with T4 endonuclease V to produce a single-strand DNA break at each CPD, electrophoresed under denaturing conditions, transferred to a membrane and sequentially hybridized with strand-specific probes. The ratio of full-length restriction fragments in the T4 endonuclease V treated and untreated samples was determined by scanning densitometry of the autoradiograms and used to determine the average number of CPDs per fragment.

2.8. Global genome repair analysis

The removal of CPDs and 6–4 photoproducts from total genomic DNA was measured using an immunoblot assay as described [7] with some modifications. Cells were irradiated with UV light (10 J/m^2) and either lysed immediately or incubated for increasing periods of time to allow for repair and then lysed. For experiments examining the effects of XPA induction, ponasterone A was added to the growth medium 24 h prior to UV irradiation and for the specified periods of time following UV irradiation. DNA was isolated, denatured and an equal amount from each sample was fixed to a Hybond nitrocellulose membrane (Amersham) using a slot blot apparatus (250 ng of DNA per slot for detection of 6–4 photoproducts and 25 ng of DNA per slot for detection of CPDs). The membranes were incubated with mouse monoclonal antibodies specific for either CPDs or 6–4 photoproducts [58]. Goat anti-mouse horseradish peroxide-conjugated secondary antibodies, enhanced chemiluminescence and autoradiography were used to detect binding of the primary antibody. After detecting the relative binding of the antibodies to CPDs or 6–4 photoproducts, the relative amount of DNA in each slot was determined by incubating the membranes with a radio-labeled DNA probe made by random priming of genomic human DNA. Scanning densitometry of the autoradiograms was used to determine the relative amount of DNA bound to each slot. The relative amount of antibody bound to each slot was divided by the relative amount of DNA bound to each slot and used to calculate the percentage of 6–4 photoproducts or CPDs removed at each time point.

2.9. Clonogenic survival analysis

Cells were plated at clonal density, incubated for 24 h, irradiated with UV doses ranging from 0 to 12 J/m^2, incubated for 10–14 days, and stained with crystal violet. Colonies were scored and surviving fractions for each dose were calculated.

2.10. Statistical analysis

The means and standard deviations were obtained using SigmaPlot (SPSS Science, Chicago, IL). Statistical analysis of the data presented in Figs. 4 and 5 used an analysis of variance (ANOVA) procedure for a $2 \times 3 \times 3$ factorial design. Post hoc comparisons of means were based on Fisher protected least significant differences procedure with a Bonferroni adjustment if the corresponding effect in the ANOVA was not found to be statistically significant at the 0.05 level.

3. Results

3.1. SSCP and sequencing analyses of human samples

DNA samples obtained from the Molecular Diagnostics Laboratory of the Department of Pathology and Laboratory Medicine at the University of Kentucky were used to identify polymorphisms in *XPA*. They derived from blood samples taken from largely unrelated patients requiring an assortment of diagnostic tests including tests for kidney or liver disease,

hemachromatosis and blood clotting deficiencies and from cadaver accident victims that could serve as potential organ donors. These samples were used to assess a random distribution of *XPA* polymorphisms and were not used to attempt to assess any association between polymorphism frequency and disease. This approach of studying a "cancer-free" population to identify novel polymorphism in repair genes has been commonly used [44,45,47,49,50]. Each of the six exons and portions of the 5′ and 3′ untranslated regions were amplified in fragments less than 364 bp using the primers described in Table 1. Each fragment was then screened for mutations by SSCP analysis. When shifted bands were identified, the bands were excised from the gel, re-amplified and sequenced and the region was re-amplified from the original patient's sample and sequenced. No consistent band shifts were identified in a 297 bp fragment of the 5′ untranslated region in 184 samples and none were identified in exons 2–5 or their adjacent intron boundaries in 189 samples (Table 2). One frequent SNP was found at the –4 position of the untranslated region (Table 2) which is a change from A to G. The G allele frequency was 0.74 (the less frequent A allele is represented in the GenBank sequence, Table 2) and the frequency of heterozygosity was 0.34 in the 134 samples. No additional sequence variations were found in the coding region of exon 1 in the 134 samples we studied. SSCP analysis revealed two rare SNPs in the coding region of exon 6 (Table 2). One is a G to A transition at codon 228 and this polymorphism codes for glutamine instead of arginine. The other is a G to T transversion at codon 234 and this polymorphism codes for leucine instead of valine. Each was identified in only one of the 184 samples examined and both individuals were heterozygotes. No sequence variations were detected by SSCP analysis in the 3′ untranslated region of exon 6.

Since SSCP analysis is less than 100% efficient in detecting point mutations, we next directly sequenced amplified fragments containing exons 4–6 in a subset of our patient samples (Table 2). Again no sequence variations were found in exons 4 and 5. Four SNPs and one single nucleotide deletion were found in intron 4 but only one, T to G at –154 5′ of exon 5, creates an

Table 2
Summary of SSCP and sequencing analyses

Region[a]	Number of samples studied	Number of variants	Position of variant[b]	Sequence variation	Amino acid change
SCCP analysis					
5′ UTR	184	0			
5′ UTR + coding region exon 1	134	1	26823 at –4 UTR	CAG to CGG	
Exon 2	189	0			
Exon 3	189	0			
Exon 4	189	0			
Exon 5	189	0			
Exon 6	184	2	5107 in codon 228	CGA to CAA	Arg to Gln
			5090 in codon 234	GTG to TTG	Val to Leu
Direct sequencing analysis					
Exon 4/intron	129	1	16590 (34 nt 3′ of exon 4)	Deletion of T	
Intron/exon 5	146	4	14724 (154 nt 5′ of exon 5)	T to G	
			14663 (94 nt 5′ of exon 5)	A to C	
			14657 (88 nt 5′ of exon 5)	C to G	
			14586 (17 nt 5′ of exon 5)	T to G	
Exon 6	103	3	5107 in codon 228	CGA to CAA	Arg to Gln
			5090 in codon 234	GTG to TTG	Val to Leu
			5036 in codon 252	CTA to GTA	Leu to Val

[a] Includes adjacent intron boundaries.
[b] Based on GenBank AL445531 (source sequence is in antisense).

alternative splice site. Each variant detected in intron 4 was identified only once and all individuals were heterozygous. In addition, direct sequencing detected the two SNPs identified in exon 6 by SSCP analysis and one additional SNP in exon 6 at codon 252. This C to G transversion results in a codon change from leucine^{252} to valine^{252}. This individual was also heterozygous.

3.2. Establishment of XP12RO transfectants containing wild type or polymorphic cDNA alleles of XPA in an ecdysone-inducible expression vector

To investigate the functional consequences of the sequence variants that we identified in exon 6 we established XP12RO-SV derived stable transfectants expressing wild type or polymorphic alleles of *XPA* cDNA. XP12RO-SV cells are homozygous for a non-sense mutation at codon 207, are devoid of TCR and GGR and are extremely sensitive to UV irradiation [59]. No XPA protein is detected by Western analysis of XP12RO-SV cells (Fig. 1) and [59]. Four stable cell lines derived from XP12RO-SV were established and used in this study: RXR (transfected with pVgRXR alone), XPAwt (transfected with pVgRXR and pIND-XPAwt), R228Q (transfected with pVgRXR and pIND-R228Q) and V234L (transfected with pVgRXR and pIND-V234L). Expression of each *XPA* allele is under the control of the *Drosophila* hormone ecdysone responsive elements. XPA expression was studied in the absence and presence of the ecdysone analog, ponasterone A (Fig. 1). Addition of increasing concentrations of ponasterone A resulted in a dose-dependent increase in the expression of XPA (data not shown). Maximal induction was achieved with 5 μM ponasterone A. In the absence of ponasterone A low amounts of XPA were detected in all three complemented cell lines which were similar to or lower than the amount found in a cell line with no known mutations in *XPA*, GM00637 (Fig. 1).

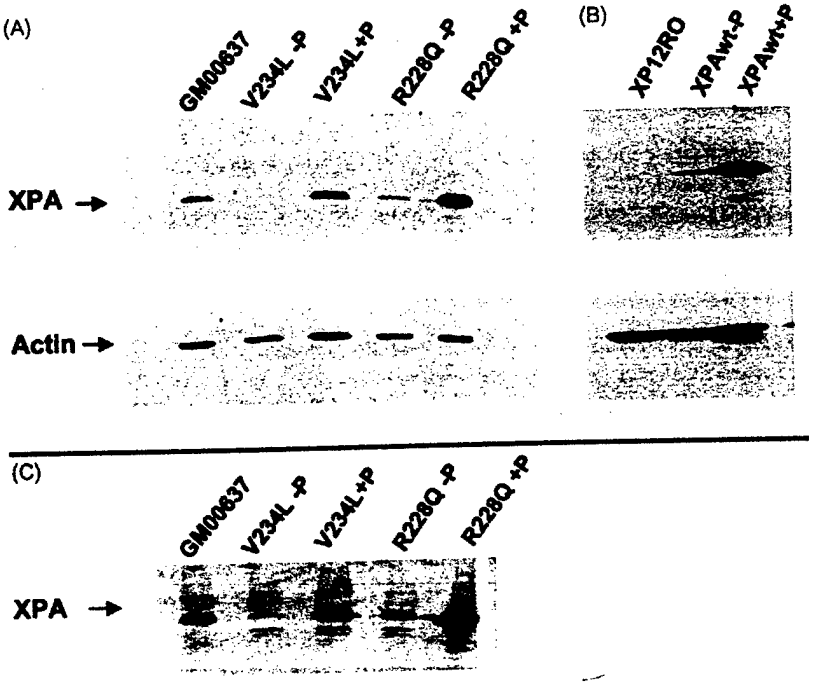

Fig. 1. Expression of wild type and polymorphic XPA protein (A–C). Cultures of GM00637, V234L, R228Q, XPAwt and RXR were lysed as described in Section 2. Cultures of V234L, R228Q and XPAwt were grown without ponasterone A (−P) or with 5 M ponasterone A (+P) added 24 h prior to lysing of the cells. Proteins from equivalent numbers of cells were separated by SDS-PAGE and analyzed by immunoblotting with anti-XPA antibodies and then anti-actin antibodies as a control. (C) Overexposure of the membrane analyzed for XPA shown in (A).

The lowest amount of expression was in V234L. Over-exposure of the autoradiogram (Fig. 1C) indicated that it was approximately 30% of the amount found in R228Q or XPAwt. Addition of 5 μM ponasterone A resulted in an approximately 10-fold increase in expression in all three XPA complemented cell lines. Expression was also examined by confocal microscopy. XPA localized to the nucleus and no changes in localization were observed after UV irradiation (data not shown). In addition, Western analysis of several independently derived clones obtained from each transfection did not reveal any significant difference in the expression or stability of wild type or polymorphic XPA proteins expressed in XP12RO-SV.

3.3. Repair in each strand of the DHFR gene

To study the effect of the exon 6 polymorphisms on repair, the removal of CPDs from each strand of the *DHFR* gene was measured in XPAwt, R228Q, and V234L in the absence and presence of ponasterone A (Figs. 2 and 3). As expected, no repair in either strand was detected in RXR or its parental cell line, XP12RO-SV (data not shown). In contrast, TCR is proficient in all XPA complemented cell lines and there is no significant difference in the kinetics of repair in the transcribed strand of the gene in any of the complemented cell lines (Figs. 2 and 3). Hence, the R228Q and V234L polymorphisms do not have a substantial impact on TCR. In addition, induction of XPA did not have a significant impact on the kinetics of TCR. Removal of CPDs from the non-transcribed strand of the *DHFR* gene was slow and inefficient in all complemented cell lines and did not significantly change when XPA was induced. Poor repair of the non-transcribed strand is likely related to the fact that the parental cell line XP12R-SV was established by transformation with SV40. A significant reduction in

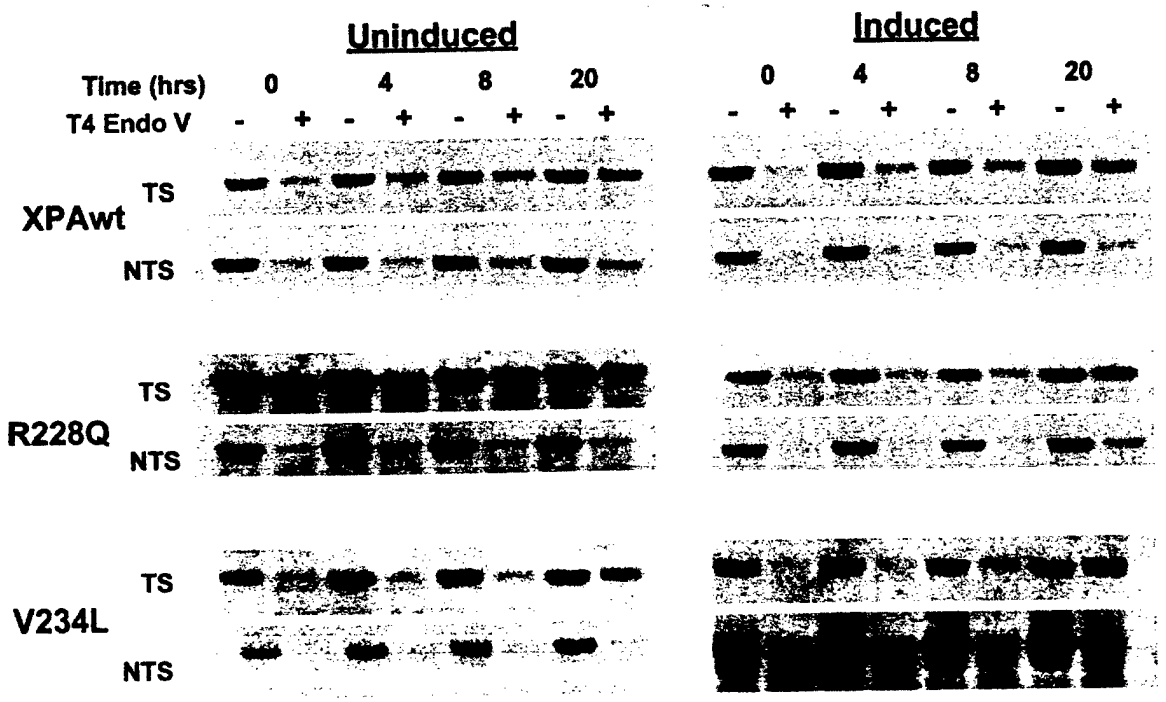

Fig. 2. Autoradiograms illustrating removal of CPDs from each strand of the *DHFR* gene in cells complemented with wild type or polymorphic alleles of XPA. Cultures of XPAwt, R228Q and V234L were grown without ponasterone A (uninduced) or with 5 μM ponasterone A (induced) for 24 h, irradiated with 10 J/m^2, and either lysed immediately (0 h) or lysed after incubation for the times indicated above the lanes. DNA was purified and analyzed using the CPD-specific enzyme T4 endonuclease V as described in Section 2. Membranes were hybridized with an RNA probe specific for the transcribed (T) strand of the *DHFR* gene, stripped and hybridized with an RNA probe specific for the non-transcribed strand (NT).

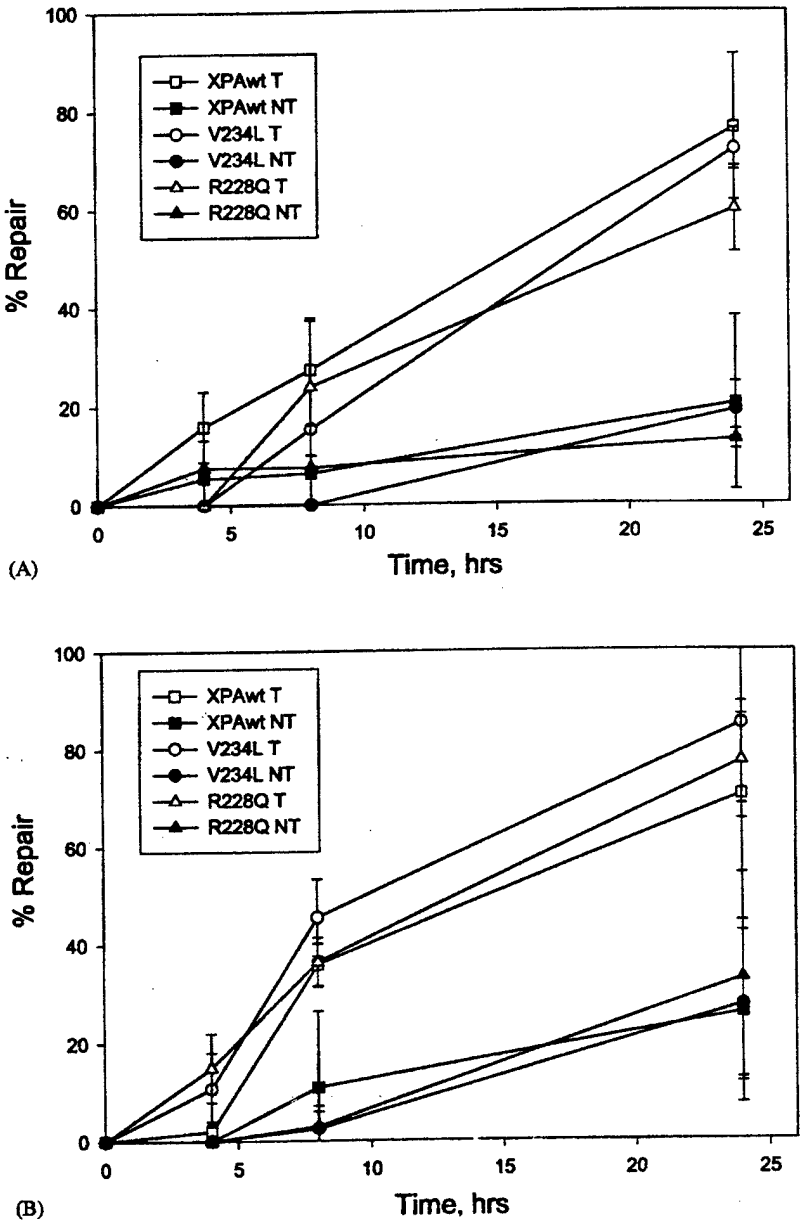

Fig. 3. Time course of removal of CPDs from each strand of the *DHFR* gene in XP12RO-SV cells complemented with wild type or polymorphic alleles of *XPA*. XPA expression was not induced (A) or induced (B) by addition of ponasterone A. Quantitative Southern hybridization (illustrated in Fig. 2) was performed using DNA samples isolated after cells were exposed to 10 J/m^2 and incubated for the specified times. The ratio of full-length restriction fragments in the enzyme-treated and untreated samples was determined by scanning densitometry of the autoradiograms and used to calculate the average number of CPDs per restriction fragment at each time point. Each value represents an average obtained from at least two independent biological experiments: XPAwt (squares), V234L (circles) and R228Q (triangles). Open symbols represent values obtained for the transcribed strand of the gene and closed symbols represent those obtained for the non-transcribed strand.

the removal of CPDs from the non-transcribed strand of the *DHFR* gene and from the genome overall has been previously found in human cell lines that have been transformed with SV40 [60].

3.4. Global genome repair of 6–4 photoproducts and CPDs

The method used to measure repair in each strand of the *DHFR* gene only measures the removal of CPDs and there is some question as to whether non-transcribed strands of expressed genes are repaired in the same way as unexpressed regions of the genome. Hence, we measured GGR of CPDs and 6–4 photoproducts using antibodies specific to each lesion. There was no substantial difference in the removal of 6–4 photoproducts or CPDs in cells containing the wild type allele of *XPA* or either of the two polymorphic alleles. The removal of CPDs from the bulk of the DNA was similar to repair of the non-transcribed strand of the *DHFR* gene; it was slow and inefficient (data not shown). In contrast, 6–4 photoproducts were rapidly removed from the total genomes of XPAwt, R228Q and V234L (Fig. 4). Interestingly, induction of XPA resulted in a roughly two-fold increase in the kinetics of 6–4 photoproduct removal in all three XPA complemented cell lines. The difference in 6–4 removal when XPA was uninduced or induced in each complemented cell line was significant ($P < 0.0001$ at 4 and 8 h and $P < 0.046$ at 24 h). In addition, when XPA was not induced, V234L removed 6–4 photoproducts somewhat less efficiently than either XPAwt or R228Q at the 4 h time point post UV-treatment (Fig. 4; $P < 0.05$). No repair was detected in XP12RO-SV or RXR (data not shown).

3.5. Cell survival

The influence of each polymorphism on colony forming ability after UV irradiation was measured (Fig. 5). As expected RXR cells were extremely sensitive to the killing effects of UV light. In contrast, XPAwt, R228Q and V234L were significantly more resistant to UV irradiation. Cells containing the polymorphic alleles survived as well as those containing the wild type allele (Fig. 5) and other human cell lines

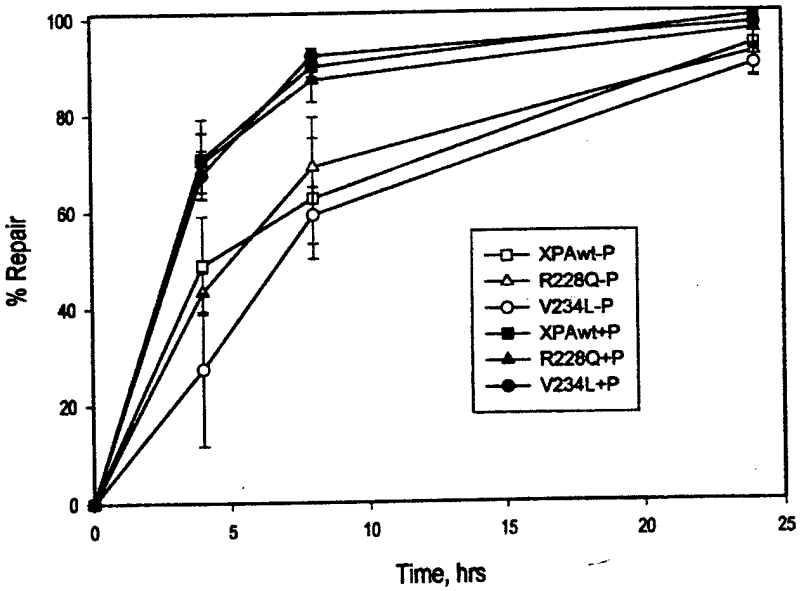

Fig. 4. Time course of removal of 6–4 photoproducts by GGR in cells complemented with wild type or polymorphic alleles of *XPA*. The percent removal of 6–4 photoproducts from the total genomic DNA of cells treated with 10 J/m^2 was determined for XPAwt (squares), V234L (circles) and R228Q (triangles) incubated in the absence (open symbols) or presence of ponasterone A (closed symbols). Each value represents the average of four independent determinations.

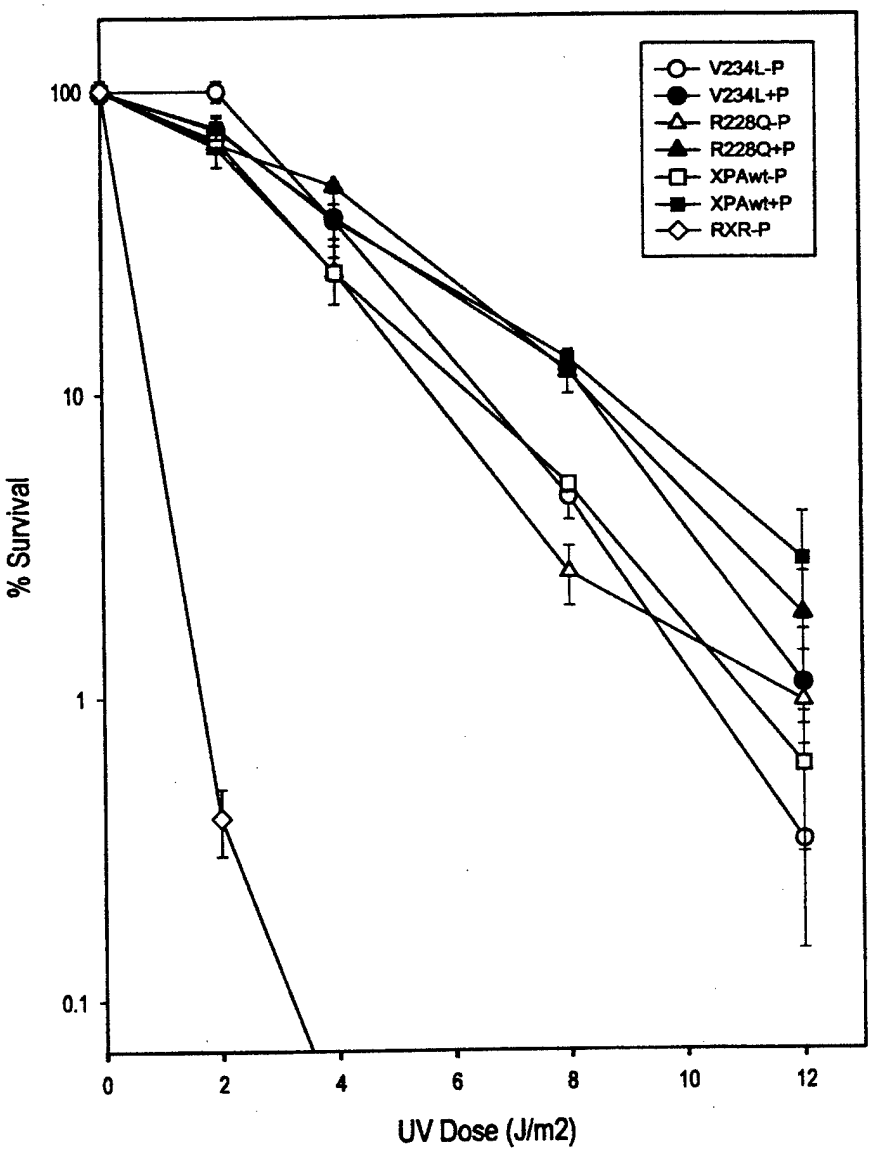

Fig. 5. UV survival based on colony forming ability of XP12RO-SV and cells derived from XP12RO-SV complemented with wild type or polymorphic *XPA* alleles. Mass cultures were grown in the absence or presence of 5 μM ponasterone A for 24 h and then plated at clonal density with or without 5 μM ponasterone A for 24 h. Cells were irradiated with increasing doses of UV, incubated for 10–14 days with or without ponasterone A and scored for colony forming ability as described in Section 2. Each value represents the average of three independent determinations. RXR with ponasterone A (open diamond), XPAwt (squares), V234L (circles) and R228Q (triangles). With the exception of RXR, open symbols represent cells grown in the absence of ponasterone A and closed symbols represent cells grown with ponasterone.

with no known defects in NER (data not shown and [59]). In addition, a small increase in cell survival occurred when XPA was induced prior to UV irradiation of all three XPA complemented cell lines (Fig. 5).

4. Discussion

We find only one frequent polymorphism in the *XPA* gene. This occurs in the 5′ untranslated region 4

nucleotides 5′ of the translation initiation codon with a frequency of heterozygosity of 0.34. The frequency of heterozygosity in our population of samples is similar to that found studying families in the UK with multiple self-healing squamous epitheliomata [61] but is significantly different from that found studying a cancer-free Polish population [49]. The basis for this difference is unclear. In addition we have identified three rare polymorphisms in the coding region of exon 6 that were identified with a frequency of 1 in 184 (SSCP analysis) or 1 in 103 (sequencing analysis). The polymorphism that we identified at codon 228 in exon 6 was also observed in a study of 35 cancer-free Caucasian individuals living in Poland [49]. Given the rarity of the allele it is unclear if the difference in the frequency of the allele in our population and in the Polish population is statistically significant. The rare polymorphisms that we identified in codons 234 and 252 in exon 6 and in intron 4 have not been reported elsewhere. Even though the polymorphisms that we identified in exon 6 are rare, it seems unlikely that they represent disease causing *XPA* mutations.

We find that the coding regions of the first five exons of the *XPA* gene are devoid of any sequence variation including those that result in silent changes. In addition, the eight variants that we identified in intron 4 or exon 6 occur at frequencies of less than 0.01. In contrast, frequent polymorphisms have been identified within the coding regions of *XPC* [50], *XPD* [44,45], *XPF* [47] and *XPG* [51]. Therefore, our results studying *XPA* appear to be unusual and may indicate that *XPA* has been restricted from genetic variation. XPA protein interacts with several other proteins including RPA, ERCC1–XPF complex and TFIIH. Protein or DNA binding domains have been mapped to each of the six *XPA* exons [62]. In addition, mutations in exons 2–6 are found in XPA patients. Hence, it is possible that an active mechanism has restricted genetic variation within the coding region of the *XPA* gene given that so much of the XPA protein appears to be critical for its ability to function properly in NER. Interestingly, some mutations in exon 6 confer milder disease symptoms than do mutations in other exons [62]. Hence, it may not be a coincidence that exon 6 was the only exon where we identified polymorphisms in the coding region of the gene if an active mechanism has restricted genetic change within the *XPA* gene. However, this is at least partially difficult to reconcile with the observations that *XPA* deficient mice are phenotypically normal with the exception of being sensitive to carcinogens and predisposed to carcinogen-induced tumors [27] and *XPA* mutations are found in humans albeit at an extremely rare frequency. Hence, variations in the *XPA* gene are not incompatible with life in mice or humans. Alternatively, *XPA* may reside in a region of the genome that is protected from genetic variation. Recently the incidence of common SNPs was found to vary across a region of the human X chromosome and six long "deserts" of low SNP incidence were identified [63]. It is possible that *XPA* resides in a "desert" region.

We find no substantial difference in TCR, GGR or cell survival following UV irradiation of cells complemented with wild type *XPA* or the Arg228Gln or Val234Leu changes in exon 6. Others have introduced wild type or mutant *XPA* into XP12RO-SV cells and have observed differences in repair and cell survival in cells containing wild type and mutant alleles [59]. Hence, it is unlikely that our inability to detect a difference in repair or cell survival between wild type and polymorphic alleles was a consequence of the system used in this investigation. In addition, we find no large differences in the repair of CPDs or cell survival after UV irradiation in cell lines containing different basal levels of XPA expression or when XPA expression was increased approximately 10-fold in the same cell line. However, we found an increase in the kinetics of removal of 6–4 photoproducts when XPA expression was induced 10-fold in cells complemented with wild type or polymorphic XPA. This may indicate that XPA protein is rate limiting in the rapid removal of 6–4 photoproducts by GGR. Interestingly, the observation that uninduced V234L cells survive UV irradiation and remove UV photoproducts as well as, or almost as well as, induced XPAwt cells may indicate that this polymorphism actually enhances the function of XPA protein. Our finding of inefficient removal of CPDs from the non-transcribed strand of the *DHFR* gene and the bulk of the genomes in the cell lines constructed in this study are consistent with previous associations of SV40 transformation and reduced GGR of CPDs [60]. However, our results may also indicate that SV40 transformation can be associated with slow TCR since most CPDs were removed from the transcribed strand of the *DHFR* gene in the complemented cell lines within 20 h after UV irradiation but the kinetics

were slow. A recent functional study of XPD polymorphic variants showed that increased sensitivity to UV-induced apoptosis was associated with homozygosity for the Asp312Asn variant [64]. There may be similar subtle differences in sensitivity to apoptosis associated with altered kinetics of repair in cells expressing the XPA variants we have studied.

Clearly the frequency of polymorphisms within the coding region of *XPA* is extremely low in the population used in this study. Our SSCP analysis was confirmed by direct sequencing of exons 4–6 in over 100 individuals. However, the large majority of individuals used in this study had no history of cancer that was known to us. A polymorphism within the *XPD* gene is more frequent in patients with basal cell carcinoma [53,54] and a polymorphism in the *XPC* gene is more frequent in patients with squamous cell carcinoma of the head and neck [65]. Hence, the investigation of populations of individuals with specific forms of skin or non-cutaneous cancer might reveal an increased frequency of certain *XPA* polymorphisms and/or new *XPA* polymorphisms. In addition, our investigation was limited to a largely Caucasian population and studies of other racial or ethnic groups might reveal differences.

Acknowledgements

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Global genome removal of thymine glycol in *Escherichia coli* requires endonuclease III but the persistence of processed repair intermediates rather than thymine glycol correlates with cellular sensitivity to high doses of hydrogen peroxide

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ABSTRACT

Using a monoclonal antibody that specifically recognizes thymine glycol (Tg) in DNA, we measured the kinetics of the removal of Tg from the genomes of wild type and repair gene mutant strains of *Escherichia coli* treated with hydrogen peroxide. Tg is rapidly and efficiently removed from the total genomes of repair proficient cells *in vivo* and the removal of Tg is completely dependent on the *nth* gene that encodes the endonuclease III glycosylase. Hence, it appears that little redundancy in the repair of Tg occurs *in vivo* at least under the conditions used here. Moreover, previous studies have found that *nth* mutants are not sensitive to killing by hydrogen peroxide but *xth* mutant strains (deficient in the major AP endonuclease, exonuclease III) are sensitive. We find that cell death correlates with the persistence of single-strand breaks rather than the persistence of Tg. We attempted to measure transcription-coupled removal of Tg in the lactose operon using the Tg-specific monoclonal antibody in an immunoprecipitation approach but were not successful in achieving reproducible results. Furthermore the analysis of transcription-coupled repair in the lactose operon is complicated by potent inhibition of β-galactosidase expression by hydrogen peroxide.

INTRODUCTION

Reactive oxygen species are formed inside cells as a consequence of exposure to ionizing radiation and a variety of chemical agents and as byproducts of normal cellular metabolism (1). Free radicals introduce a large number of modifications to DNA including alterations of purine and pyrimidine bases, the deoxyribose sugar and cleavage of the phosphodiester backbone. Oxidative DNA damage has been implicated in the etiology of human diseases ranging from cancer (2) to aging (3).

One of the major stable modifications of thymine produced by oxidative agents is thymine glycol (Tg) which is formed by oxidation of the 5,6 double bond of thymine. It is primarily removed by base excision repair (BER) in *E. coli*, yeast and mammalian systems (1,4-6). In *E. coli*, endonuclease III (endo III) is an oxidative damage DNA glycosylase with somewhat broad substrate specificity that includes Tg (7-9). In addition to its glycosylase activity, it has an associated lyase activity that cleaves the DNA backbone by β-elimination. This produces an α,β-unsaturated aldehyde attached to the 3' end of a single strand break that must be repaired prior to DNA polymerization. *E. coli* possesses two 5' AP endonucleases, exonuclease III (exo III) and endonuclease IV (endo IV). Exo III is the major AP activity and cleaves 5' to the site of base loss producing the required 3' OH for DNA repair synthesis. The single base gap is filled in by DNA

polymerase I and sealed by DNA ligase. Endo IV also acts as a 5' AP endonuclease and while it represents less than 10% of the constitutive AP endonuclease activity in the cell, it is induced 10 to 20-fold by paraquat (10) or nitric oxide (11).

Tg poses a strong block to *E. coli* Klenow DNA polymerase (12) and eukaryotic α DNA polymerase (13). Its presence in single stranded phage reduces or inactivates the transfection efficiency of phage DNA into host cells (14). While Tg is only weakly mutagenic, this could be a consequence of accurate translesion synthesis by specialized DNA polymerases (15,16) or recombination. The ability of Tg to block DNA polymerases and inactivate phage DNA suggests that it can contribute to oxidative damage-induced lethality in cells (17-19). However, endo III is encoded by the *nth* gene but quite interestingly, *nth* mutants are not hypersensitive to oxidative agents such as hydrogen peroxide or ionizing radiation (20). This could be explained by redundancy in the repair of Tg. An additional glycosylase that recogniz es Tg, endonuclease VIII (endo VIII), has been identified in *E. coli* and characterized (21-23). Endo VIII is encoded by the *nei* gene and *nth nei* double mutants are hypersensitive to hydrogen peroxide and ionizing radiation. Furthermore, Tg has been reported to be a substrate for the UvrABC-mediated nucleotide excision repair system in *E. coli* (24,25). Hence, there may be redundancy in the removal of Tg in cells.

Strains with mutations in *xth*, deficient in the major AP endonuclease, exonuclease III, are very sensitive to treatment with hydrogen peroxide (26-29). As suggested by Demple et al. (26), the sensitivity of *xth* strains may at least in part be caused by the persistence of repair intermediates generated by the glycosylase and lyase activities of endo III or abnormal termini generated directly by hydrogen peroxide. This is supported by the observation that *xth* mutants exposed to hydrogen peroxide accumulate large numbers of single strand breaks in their chromosomes (30).

Transcription-coupled repair (TCR) is a subpathway of repair whereby lesions are removed more rapidly or more efficiently from the transcribed strands of expressed genes than from the nontranscribed strands (31,32). This feature of repair has been clearly demonstrated to be a subpathway of nucleotide excision repair (NER) in *E. coli* (33), yeast (34,35) and mammalian cells (36-38). It operates on a wide spectrum of bulky adducts that generally block transcription elongation. While the precise mechanism is unknown, an early event likely involves blockage of the RNA polymerase complex at lesions present in the transcribed strands of expressed genes.

It has been suggested that BER pathways can also be coupled to transcription and Tg has been found to be removed more rapidly from the transcribed strands of expressed genes in yeast (34) and mammalian cells (39,40). However, there has been no direct genetic demonstration of a role of

BER in TCR. While it has been reported that 8-oxoguanine is a substrate for TCR in mammalian cells, the glycosylase Ogg1 which is required for the removal of 8-oxoguanine from nontranscribed sequences, is not required for its removal from transcribed sequences (41). Moreover, 8-oxoguanine (42) and Tg (43) are not efficient blocks to RNA polymerase progression. Hence, it is unclear if TCR is a bona fide sub-pathway of a glycosylase mediated BER pathway or whether Tg is removed in a transcription-dependent manner in *E. coli*.

In the present study, the removal of Tg from the genomes of wild type and repair gene mutant strains of *E. coli* treated with hydrogen peroxide was measured using a monoclonal antibody that specifically recognizes Tg in DNA. Tg is rapidly and efficiently removed from the total genomes of repair proficient bacterial cells *in vivo*. Investigation of BER and NER gene mutant strains indicates that the removal of Tg is completely dependent on the *nth* gene that encodes the endo III glycosylase. Hence, it appears that little redundancy in the repair of Tg occurs *in vivo* at least under the conditions used here. Previous studies have found that *nth* mutants are not sensitive to killing by hydrogen peroxide (20) but *xth* mutants (deficient in the major AP endonuclease, exonuclease III) are sensitive (26-29). In the present study, cellular sensitivity to hydrogen peroxide correlates with the persistence of strand breaks rather than the persistence of Tg. Hence, it appears that repair intermediates or abnormal termini directly induced by oxidative agents contribute more to cell killing than do Tg adducts. We also attempted to measure TCR of Tg in the *lac* operon using

the Tg-specific monoclonal antibody in an immunoprecipitation approach but were not successful in achieving reproducible results. Furthermore the analysis of TCR in the *lac* operon is complicated by potent inhibition of β-galactosidase expression by hydrogen peroxide.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The bacterial strains used in this work are derivatives of *E. coli* K-12 and are listed in Table 1. The MGM strain is a derivative of W3110. The other mutant strains are derivatives of AB1157. The AB1157 strain and its derivatives were grown at 37°C in Difco Bacto minimal broth Davis (MB) supplemented with 0.4% glucose, the appropriate antibiotic, 200 μg/ml each of arginine, histidine, leucine, proline and threonine, and 10 μg/ml thiamine (complete medium). W3110 and MGM were grown at 37°C in MB supplemented with 0.4% glucose and 2 μg/ml thymine (complete medium).

Measurement of Thymine Glycol

Cultures were grown to saturation in complete medium, diluted 1:50 in complete medium, and grown to an absorbance of 0.2 at 650 nm. To induce transcription of the *lac* operon, isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM and growth was continued to an absorbance of 0.55. Hydrogen peroxide was added and the cultures were incubated on ice for

10 minutes. To terminate the hydrogen peroxide treatment, cells were collected by filtration on 0.45 µm Millipore membrane filters, washed with MB and resuspended at the same density in complete medium containing 1 mM IPTG. Cells were either lysed immediately, or incubated in complete medium for increasing periods of time at 37°C to allow repair and then lysed. For each time point, 20 ml samples were collected on ice and quickly chilled by the addition of 20 ml prechilled 2X NET (200 mM NaCl; 20 mM EDTA, pH 8.0; 20 mM Tris, pH 8.0). Cells were collected on 0.45 µm Millipore filters, resuspended in 700 µl TE (10 mM Tris, pH 8.0; 1 mM EDTA pH 8.0) and lysed by the addition of 1 mg lysozyme for 30 minutes at 37°C. Samples were treated with RNase A at 200 µg/ml for 30 minutes, and then incubated overnight at 37°C with proteinase K at 100 µg/ml and 0.5% Sarkosyl sulfate. Samples were extracted with phenol and DNA was precipitated with 2.5 M ammonium acetate and 0.6 volumes isopropyl alcohol. Purified DNA was resuspended in TE and incubated with *Apa*I and *Sst*II (BRL, Life Technologies, Grand Island, NY) overnight.

The introduction and removal of Tg was quantified using a monoclonal antibody that recognizes Tg in DNA in an enzyme linked immunosorbant assay (ELISA) as described by Leadon (44) as follows. Polystyrene microtiter plates were incubated with 20 µg/well protamine sulfate for 90 minutes at room temperature. Protamine sulfate was removed by washing the plates 3 times with distilled water. OsO_4-modified DNA in 50 µl phosphate-buffered saline (PBS) was adsorbed to each well by drying the DNA for 16 hours at 37°C. The plates were

then washed with PBS containing 0.05% Tween 20 and 1% heat-inactivated horse serum (PBS/Tween/hs), and incubated with 1% heat-inactivated horse serum for 4 hours at room temperature. The solution was removed and plates were washed with PBS/Tween/hs. The monoclonal antibody with or without competitor DNA (DNA from bacterial cells treated with hydrogen peroxide) was added to each well and incubated for 90 minutes at 37°C. Wells were washed and incubated with alkaline phosphatase conjugated rabbit anti-mouse IgG for 90 minutes at 37°C. Unbound antibody was removed and 0.2 ml 1 mg/ml p-nitrophenylphosphate in 100 mM glycine, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, pH 10.4 was added to each well and incubated at 37°C for 1-2 hours. The amount of primary antibody bound in each well was determined by spectrophotometric analysis of the production of p-nitrophenol.

Clonogenic Survival

Cultures were grown to saturation in complete medium, diluted 1:50 in complete medium, and grown to an absorbance of 0.4 at 650 nm. Hydrogen peroxide was added and cells were incubated on ice or at 37°C. Samples (10 μl) were taken after cultures were incubated for 7.5 or 15 min, diluted in 1.8 ml pre-chilled MB and placed on ice. Serial dilutions were spread on LB plates and incubated for 24 h at 37°C.

Measurement of β-Galactosidase

Cultures were grown to saturation in complete medium, diluted 1:50 in complete medium, grown to an absorbance of 0.4 at 650 nm and incubated with hydrogen peroxide for 10 minutes on ice or at 37°C. Cells were then collected by filtration on 0.45 μm Millipore filters, washed with MB and resuspended in complete MB medium and 1mM IPTG. Samples (500 μl) were taken after 30, 60, 90, and 120 min of incubation at 37°C. β-galactosidase activities were determined as described by Miller (45) except that cells were permeabilized by freezing samples in a dry ice/ethanol bath and then quickly thawing them at 37°C (three cycles).

Examination of Persisting Strand Breaks in Wild Type and Mutant Strains Treated with Hydrogen Peroxide

Cultures were expanded, treated with hydrogen peroxide, incubated for increasing times to allow repair and the DNA was purified and treated with restriction enzymes as described in the "measurement of thymine glycol" section. Two samples of DNA from each time point were examined for each experiment using wild type, *nfo, xth* or *nfo xth* mutant strains. One sample from each time point was denatured for 3 minutes at 100°C and the other sample was not heat denatured. All samples were incubated on ice for 15 minutes and then electrophoresed overnight in non-denaturing 1% agarose gels. Gels were stained with either ethidium bromide or GelStar (FMC Bioproducts, Rockland, ME), photographed and the DNA from the gel transferred to a membrane, hybridized

with ^{32}P-labeled probe, washed and exposed to x-ray film. RNA probes were made using the pZH10 plasmid that contains a portion of the lactose operon (33).

RESULTS

Tg is induced in a linear, hydrogen peroxide dose-dependent manner and cell death is reduced by treatment of cells with the drug at 0°C.

Since one of the goals of this study was to measure the removal of Tg from each strand of the *lac* operon, cells were treated with increasing concentrations of hydrogen peroxide to determine the concentration that would introduce approximately 2 Tgs per 6.6 kb of DNA in each strand. This lesion frequency has proven optimal for the investigation of TCR of UV-induced damage in *E. coli* (33,46). Wild type cells were exposed to increasing concentrations of hydrogen peroxide ranging from 25 to 200 mM, for 10 minutes at 0° or 37°C. DNA was isolated from cells, treated with restriction enzymes, and the ELISA method was used to determine the frequency of Tg introduced by each dose of hydrogen peroxide. Treatment of cells with increasing concentrations of hydrogen peroxide resulted in a linear increase in the number of Tg adducts introduced into DNA and treatment of cells with 200 mM hydrogen peroxide resulted in the introduction of ~ 2 Tg lesions per 6.6 kb of DNA/strand (Fig.1A). Similar results were obtained when cells were treated with hydrogen peroxide on ice or at 37°C.

Cell survival was compared after treatment of wild type cells with 200 mM hydrogen peroxide on ice or at 37°C. Substantially more cell death was observed when cells were treated at 37°C (Fig. 1B). To minimize the lethal effects of the drug, subsequent experiments to examine the removal of Tg were carried out by exposing cells to hydrogen peroxide on ice.

Global Genome Removal of Tg is completely dependent on the *nth* gene.

The removal of Tg from the total genomes of wild type and various DNA repair mutant strains was measured. Cells were treated with hydrogen peroxide at 0°C and lysed immediately or after incubating for increasing periods of time at 37°C to allow repair. Results obtained using two wild type strains, AB1157 and W3110, indicate that Tg is rapidly removed from total DNA; 50% of the initial Tg damage was removed within 10 minutes and 80% was removed within 40 minutes after hydrogen peroxide treatment (Fig. 2A). Repair was examined in three different single BER mutant strains. The *nth* mutant strain is deficient in the glycosylase, endo III. The *xth* mutant strain is deficient in exo III that provides the major AP endonuclease activity. The *nfo* mutant strain is defective in endo IV that provides a minor AP endonuclease activity. Only the *nth* mutant defective in endo III showed reduced repair of Tg (Fig. 2A). Moreover, no removal of Tg was observed in the absence of endo III. Repair in the *xth* or *nfo* mutant strains resembled that found in wild type strains, it was fast and very efficient. In addition, global genome repair was examined in several strains containing different combinations of double mutations (Fig. 2B). In the double AP

endonuclease mutant strain, *xth nfo*, repair was very similar to that found in wild type cells. In contrast, consistent with the single mutant data, double mutant strains containing a mutation in *nth* were completely deficient in the removal of Tg.

Global genome removal of Tg is unaffected in an NER deficient strain

The UvrABC damage recognition and incision complex that functions in NER in *E. coli* has been found to recognize Tg *in vitro* (24,25). To test whether NER plays a major role in the removal of Tg from cells *in vivo*, repair was measured in a *uvrA* mutant strain. First, the UV sensitivity of the *uvrA* strain was tested and found to exhibit the expected UV sensitive phenotype (data not shown). However, the removal of Tg was similar to that observed in the wild type strains (Fig. 2A).

Hydrogen peroxide inhibits induction of β-galactosidase

Measurement of the removal of Tg from each strand of the *lac* operon was attempted using the Tg specific monoclonal antibody in an immunoprecipitation assay. The method uses the Tg antibody to physically separate DNA fragments containing Tg from DNA fragments free of Tg. While this assay has been used to measure repair in the individual strands of active genes in yeast and mammalian cells, reproducible results were not obtained using this approach in *E. coli*. Perhaps the immunoprecipitation analysis was complicated by the higher copy number of the bacterial genome resulting in renaturation of the DNA before

stable antibody-Tg complexes were formed or the precipitation reaction used to separate antibody bound from antibody free fractions may have caused dissociation of the antibody from the DNA.

The affect of hydrogen peroxide treatment on expression of the *lac* operon was also studied. Cultures were treated with different concentrations of hydrogen peroxide for 10 min on ice, 200 mM hydrogen peroxide for 10 min at 37°C, or 40 J/m^2 UV light and β-galactosidase activities were measured at the times shown (Fig. 3). Induction of β-galactosidase was greatly inhibited by treatment with hydrogen peroxide. 40 J/m^2 UV light was included for comparison since there is sufficient transcription of the *lac* operon at this dose for TCR to occur (33,46). However, most concentrations of hydrogen peroxide significantly reduced β-galactosidase induction to levels well below those observed with 40 J/m^2 of UV light. Hence, it is likely that the concentrations of hydrogen peroxide required to measure TCR using established methods inactivate transcription of the operon.

The generation of repair intermediates by endo III and perhaps other glycosylases contributes to hydrogen peroxide-induced cell death

The sensitivity of wild type and mutant strains to the killing effects of hydrogen peroxide was examined (Fig. 4). Cells were treated with 200 mM hydrogen peroxide at 0°C, aliquots were taken at 7.5 and 15 minutes after hydrogen peroxide treatment, and the appropriate dilutions were plated on LB agar plates. As has been found previously, the *nth* mutant strain was not

sensitive to killing by hydrogen peroxide (20) while the *xth* mutants deficient in the major AP endonuclease activity were very sensitive (26-29) (Fig. 4). Sensitivity was even greater in the double *nfo xth* mutant than in the single *xth* mutant strain, although the *nfo* mutation alone did not exhibit a sensitivity different from that of wild type. Hence, as previously shown (20,26-29), inactivation of the major AP endonuclease activity results in significant cell killing by treatment with hydrogen peroxide. Cell death is further increased by inactivation of both the major and minor AP endonuclease activities. In contrast, inactivation of the endo III glycosylase that we have shown is required for repair of Tg does not result in enhanced sensitivity to hydrogen peroxide.

The repair and cell survival data are consistent with a model that hydrogen peroxide-induce cell death is at least in part caused by the persistence of abnormal 3' termini (repair intermediates) rather than the persistence of Tg. Abnormal 3' termini can be generated by the direct action of oxidative agents or during the repair of Tg and other oxidative damage by bifunctional DNA glycosylases. To test the model, the effect of exonuclease III and endonuclease IV in processing single-strand termini was investigated by examining the integrity of DNA isolated from wild type and mutant cells at different times after treatment with hydrogen peroxide. Two equivalent samples of DNA from each repair time point were examined: one was not denatured and the other one was heat denatured for 3 minutes at 95°C. The samples were electrophoresed in parallel using non-denaturing agarose gels, transferred to a membrane, and probed with

a ^{32}P-labeled RNA probe specific for the *lac* operon. Single-strand DNA migrates with a faster mobility compared to duplex DNA under the conditions used in these experiments and DNA possessing single-strand breaks does not appear as full length in the denatured samples. It appears, instead, as a smear due to fragmentation into smaller segments.

The DNA from wild type cells and the *nfo* mutant strain had minimal single strand breaks at each time point examined (Fig. 5). In contrast, denatured DNA from each time point for the *xth* mutant strain and the *xth nfo* double-mutant strain possessed large numbers of breaks. This was observed as a conversion of the full-length fragment present in the non-denatured samples to a smear below the position of the full-length single strand DNA in the denatured lanes at the different time points (Fig. 5). Hence, it appears that in the absence of exonuclease III, strand breaks likely representing abnormal 3' termini generated by glycosylase activities persist after treatment of cells with hydrogen peroxide. In the wild type and *nfo* mutant strain, little smearing is observed below the full-length single strand DNA even at the 0 point and early repair time points. This likely reflects the coordination of glycosylase activity with post-incision events otherwise smearing should have been observed in the early times that converts to full-length fragments in the later time points. Thus the formation of strand breaks at this dose of hydrogen peroxide is more likely to be a consequence of repair rather than the direct action of reactive oxygen species.

DISCUSSION

Various aspects of the removal of Tg in *E. coli* were examined *in vivo*. The approach involved using a monoclonal antibody that specifically recognizes Tg in DNA. In establishing conditions for treating cells with hydrogen peroxide, we found that treating cells on ice resulted in a significant reduction in cell death compared with treating them at 37°C. This result is consistent with two previous reports that the cytotoxicity of hydrogen peroxide is diminished at low temperatures in yeast (47) and in mammalian cells (48). However, the levels of Tg introduced at the two different temperatures were comparable, results similar to those obtained in a previous study of yeast (47). Hence, the temperature-dependent difference in hydrogen peroxide induced cytotoxicity in *E. coli* does not correlate with differences in the levels of Tg. This may reflect temperature-dependent differences in the direct introduction of other types of DNA damage by hydrogen peroxide, such as clustered DNA lesions, or differences in the production of repair intermediates that may result in a greater frequency of lethal double-strand breaks at 37°C.

The removal of Tg is rapid and efficient in wild type strains even at the relatively high lesion frequency introduced in these experiments, ~2 Tg per 6.6 kb of DNA per strand. Previous studies of Tg removal in *E. coli* have used biochemical assays to measure repair *in vitro* (8,9) or have used more indirect approaches such as measuring the transfection efficiency of oxidatively damaged phage DNA (19). Here, the introduction and removal of Tg was directly measured

in cells. There are at least two mechanisms that produce damage in *E. coli* exposed to hydrogen peroxide and two modes of cell death have been observed (29). "Mode-one" killing occurs at low doses of hydrogen peroxide and reaches a maximum at 1 to 3 mM hydrogen peroxide. It requires active metabolism and has been shown to occur through the Fenton reaction *in vivo* and *in vitro* (28,49). "Mode-two" killing occurs at higher doses (> than 20 mM) and can occur in the absence of metabolism. The dose response for the generation of lesions that result in "mode-two" killing is linear with respect to the concentration of hydrogen peroxide while the dose response for "mode-one" killing is more complicated (28,50). The repair and survival data presented here likely relate to "mode-two" killing. However, it would be surprising if the removal of Tg from wild type and repair gene mutant strains would differ at lower doses of hydrogen peroxide or under conditions where cells are exposed to the drug at 37°C rather than 0°C.

Biochemical studies have demonstrated that endo III acts as a glycosylase to recognize and remove Tg from DNA (7). However, many years ago, studies of *nth* mutants lacking endo III found that they are no more sensitive to treatment with oxidative agents than wild type strains (20), while *xth* mutant strains were found to be very sensitive (26,27). These observations have provided an interesting conundrum. Either Tg is not a lethal lesion or back-up systems exist that can also remove Tg. The identification of an additional glycosylase, endonuclease VIII that also recognizes Tg (21-23) and observations that the UvrABC nucleotide excision repair system recognizes Tg *in vitro* (24,25) suggest

redundancy in the removal of Tg from cells. However, in the present study, the removal of Tg *in vivo* is completely dependent on endo III, little if any removal of Tg is detected in *nth* deficient strains. In addition, no deficiency in the removal of Tg was found in a *uvrA* mutant strain. These results suggest that NER does not serve a major role in the removal of Tg *in vivo* at least for the dose of hydrogen peroxide used in our experiments. Furthermore, while the removal of Tg was not examined in a *nei* mutant strain defective in endo VIII, the complete absence of Tg removal in *nth* mutant strains also suggests that endo VIII does not serve a major back-up role in the processing of Tg *in vivo*. It has been shown that endo VIII is responsible for only 10% of the nicking activity of Tg-containing DNA substrates in extracts from wild type cells (23). Thus, endo VIII may not be able to make a measurable contribution to the removal of the large numbers of Tg induced by the dose of hydrogen peroxide used here. In addition, the removal of Tg is not affected in *xth*, *nfo* and *xth nfo* mutant strains. Hence, the extent or kinetics of removal of Tg by endo III *in vivo* is not affected by deficiencies in the ability to carry out the subsequent processing of the 3' termini generated by endo III.

This study indicates that Tg is not a lethal lesion in the *E. coli* genome in cells treated with high concentrations of hydrogen peroxide. A diagram illustrating how the processing of hydrogen peroxide-induced damage can contribute to cell death is presented (Fig. 6). The *nth* mutant cells are completely deficient in removing Tg from their genomes but they are not sensitive to doses of hydrogen

peroxide that induce the formation of substantial levels of Tg. In contrast, previous studies (26-29) and ours find that *xth* mutant cells are highly sensitive to hydrogen peroxide. In addition, we find that the *xth* mutant strains are proficient in the removal of Tg from their genomes but are deficient in the processing of strand breaks or abnormal termini. These results indicate that the abnormal termini generated by endonuclease III, other glycosylases or directly by reactive oxygen species represent lethal lesions induced by hydrogen peroxide. These results are consistent with other studies that have provided evidence that the lethal damage induced by hydrogen peroxide is 3'-blocked termini or repair intermediates (30,51,52).

We were unable to measure TCR in the *lac* operon. The use of the Tg-specific monoclonal antibody in an immunoprecipitation approach to fractionate Tg containing *lac* fragments from those free of Tg proved not to be amenable to these studies. The investigation of TCR was also complicated by observations that treatment with hydrogen peroxide resulted in a significant reduction in β–galactosidase induction. Levels were significantly below those found after treatment with 40 J/m^2 UV light. Hence, even if the antibody fractionation approach had been successful, it is likely that the levels of transcription inhibition would have precluded the ability to measure TCR. Given the genetic tractability of *E. coli* to study the specific involvement of BER in TCR of oxidative damage, future investigation is warranted to determine if oxidative damage is repaired by TCR in *E. coli* and if so, what roles different repair pathways play in it.

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FIGURE LEGENDS

Figure 1. Tg is induced in a linear and temperature-independent fashion but cells are more sensitive when treated with hydrogen peroxide at 37°C. **(A)** Wild type cells (AB1157) were treated with the concentrations of hydrogen peroxide shown for 10 min at 0°C (closed circle) or 37°C (open circle), the DNA was isolated and used in an ELISA assay to determine the Tg content as described in Materials and Methods. **(B)** Wild type cells (AB1157) were treated with 200mM hydrogen peroxide at 0°C (closed circle) or 37°C (open circle) for 10 min and serial dilutions were spread on LB plates, incubated for 24 h at 37°C, and scored for colony forming ability.

Figure 2. Time course of removal of Tg from wild type and repair deficient cells. Cells were exposed to 200 mM hydrogen peroxide for 10 min at 0°C, the drug was removed and cells were incubated for the times indicated at 37°C to allow repair. The DNA was isolated, treated with restriction enzymes, purified and used in an ELISA assay to determine the Tg content at each time point. **(A)** Results obtained studying wild type (AB1157 and W3110) (closed circle) and strains with a single mutant repair gene: *xth* (closed square), *nfo* (open triangle), *uvrA* (open diamond) and *nth* (open hexagon). **(B)** Results obtained studying strains with mutations in two different repair genes: *xth nfo* (closed circle), *nth xth* (open triangle) and *nth nfo* (closed square).

Figure 3. Treatment with hydrogen peroxide inhibits induction of β-galactosidase from the *lac* operon. Log phase cultures of wild type AB1157 and W3110 were treated with the indicated concentrations of hydrogen peroxide at 0°C or 37°C, 40 J/m^2 UV light or not treated (control). Cells were washed with MB and resuspended in complete medium containing 1 mM IPTG. Aliquots were taken at the times indicated and β-galactosidase activities were measured. No treatment of cells (closed circle), 40 J/m^2 UV (open circle), 25 mM (closed square), 50 mM (open square), 100 mM (closed triangle), 200 mM (open triangle) hydrogen peroxide at 0°C, 20 mM hydrogen peroxide at 37°C (closed inverted triangle).

Figure 4. *xth* mutant strains are highly sensitive to treatment with 200 mM hydrogen peroxide at 0°C. Log phase cultures were treated with 200 mM hydrogen peroxide at 0°C and aliqots were taken after incubation for the times shown. Serial dilutions were spread onto LB plates, incubated at 37°C for 24 h and scored for colony forming ability. Wild type (closed circle), *nfo* (closed triangle), *nth* (closed inverted triangle), *nth nfo* (open inverted triangle), *xth* (closed square), *xth nth* (closed hexagon), and *xth nfo* (closed diamond).

Figure 5. Single strand breaks persist in *xth* strains after treatment with hydrogen peroxide. Cells were treated with 200 mM hydrogen peroxide at 0°C for 10 min, the drug was removed and cells were incubated at 37°C for the indicated times to allow repair. DNA was isolated, treated with restriction enzymes and purified. Two samples were taken at each time point. One sample was denatured by

incubation at 100°C for 3 min and the other sample was not denatured. All samples were electrophoresed under non-denaturing conditions and the DNA was transferred to a membrane and the fragment containing the *lac* operon detected using radio-labeled probe. The arrows indicate the position of full-length single stranded *lac* containing fragment.

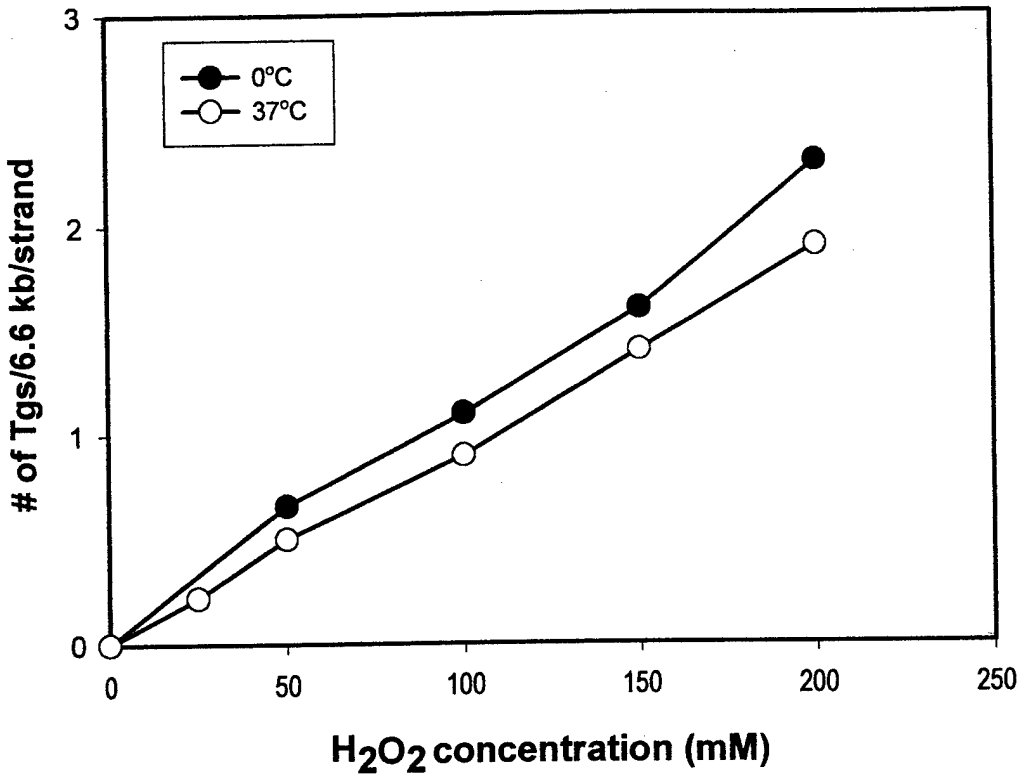
Figure 6. Diagram of the initial steps of base excision repair (reviewed in (5,6)) to illustrate how the processing of different lesions influences cell death. One strand of DNA is represented containing a Tg. Endo III first catalyzes the breakage of the glycosyl bond between the base and the DNA sugar-phosphate backbone and releases Tg. This is followed by the AP lyase activity of endo III that produces a 3' α,β-unsaturated aldehyde and a 5' phosphate. Exo III then cleaves the phosphodiester bond 5' to the AP site producing a normal 3' terminus and a gap of one nucleotide. No significant removal of Tg is found in *nth* mutant strains but they survive exposure to high doses of hydrogen peroxide as well as wild type cells. Hence, the presence of Tg in DNA does not correlate with cell death. In contrast, *xth* mutant strains efficiently remove Tg and the removal of Tg by endo III or the processing of other lesions induced by hydrogen peroxide results in the formation of strand breaks containing abnormal 3' termini. The sensitivity of *xth* mutants to high concentrations of hydrogen peroxide is likely a consequence of the inability to process repair intermediates.

Table 1. Bacterial strains used in this study.

Strain	Relevant genotype	Source
AB1157	Wild type	R. Cunningham
BW415	Δ*nth*	R. Cunningham
BW9109	Δ*xth*	R. Cunningham
BW435	Δ*xth* Δ*nth*	R. Cunningham
RPC500	*nfo*-1::Tn5	R. Cunningham
RPC501	Δ*xth nfo*-1::Tn5	R. Cunningham
BW534	*nth*-1::Tn5 *nfo*-1::Tn5	R. Cunningham
W3110	Wild type	R. Bockrath
MGM	*uvrA*277::Tn10	R. Bockrath

Fig. 1

A


0°C
37°C
# of Tgs/6.6 kb/strand
0
1
2
3
0
50
100
150
200
250
H2O2 concentration (mM)

B

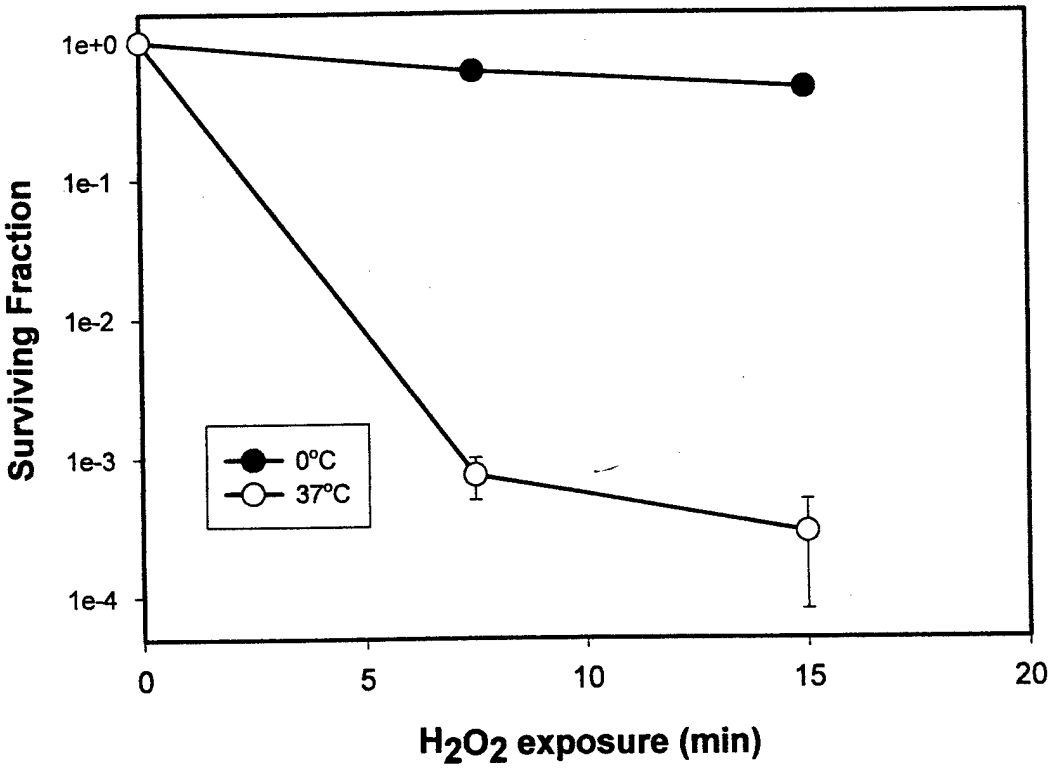

Surviving Fraction
1e+0
1e-1
1e-2
1e-3
1e-4
0°C
37°C
0
5
10
15
20
H2O2 exposure (min)

Fig. 2

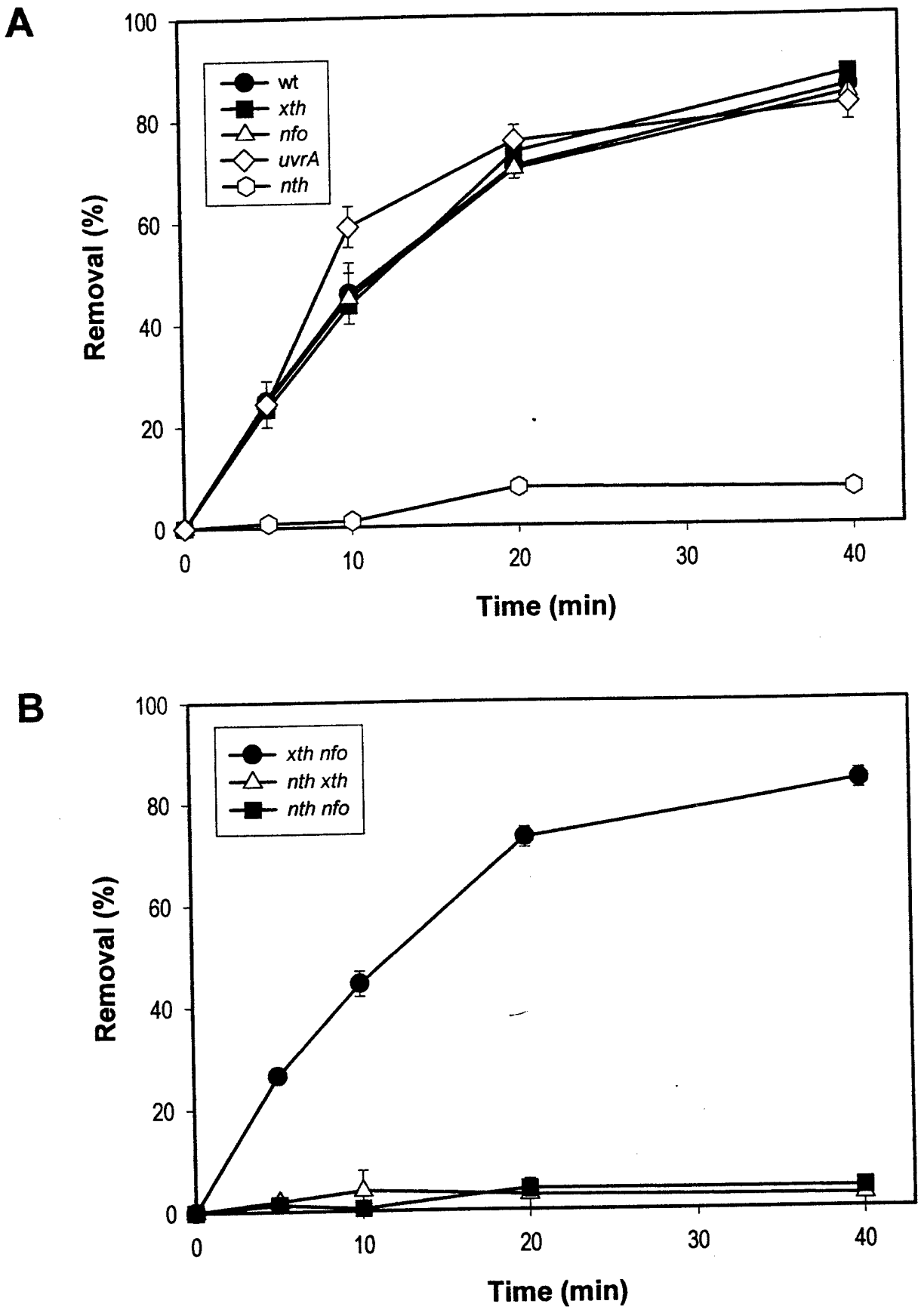

A
100
80
60
40
20
0
Removal (%)
wt
xth
nfo
uvrA
nth
0
10
20
30
40
Time (min)
B
100
80
60
40
20
0
Removal (%)
xth nfo
nth xth
nth nfo
0
10
20
30
40
Time (min)

Fig. 3

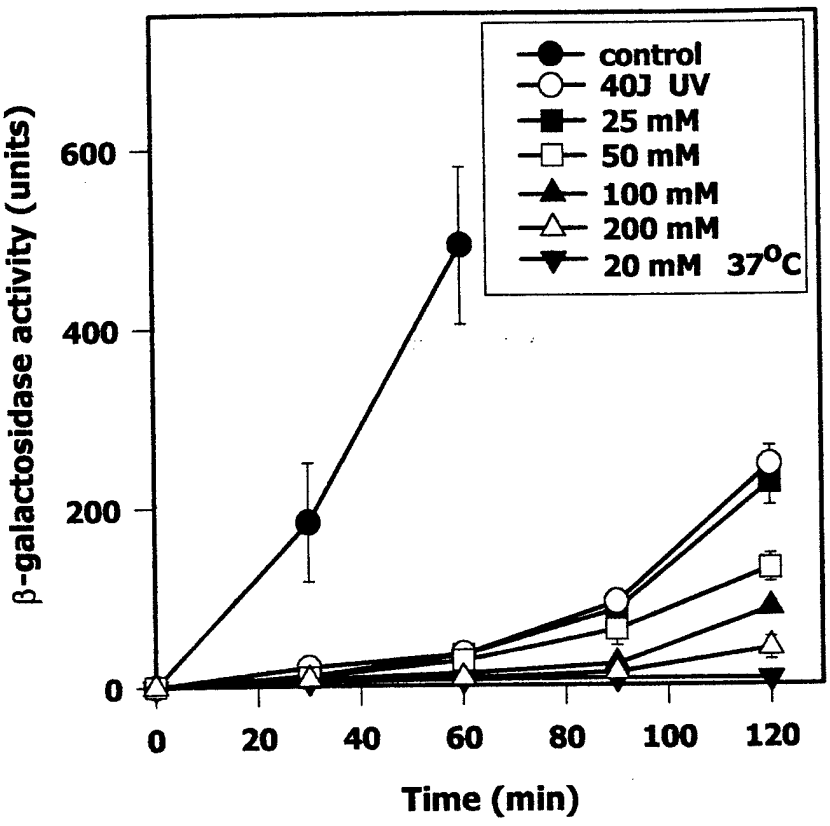

control
40J UV
25 mM
50 mM
100 mM
200 mM
20 mM 37°C
β-galactosidase activity (units)
600
400
200
0
0
20
40
60
80
100
120
Time (min)

Fig. 4

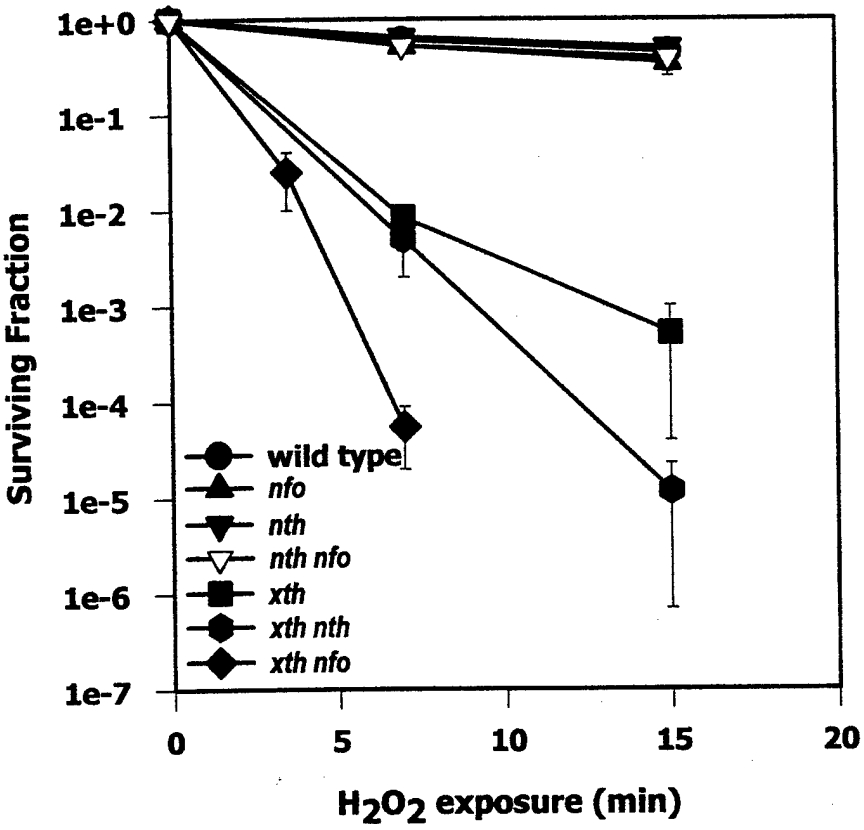

1e+0
1e-1
1e-2
1e-3
1e-4
1e-5
1e-6
1e-7
Surviving Fraction
wild type
nfo
nth
nth nfo
xth
xth nth
xth nfo
0
5
10
15
20
H2O2 exposure (min)

Figure 5

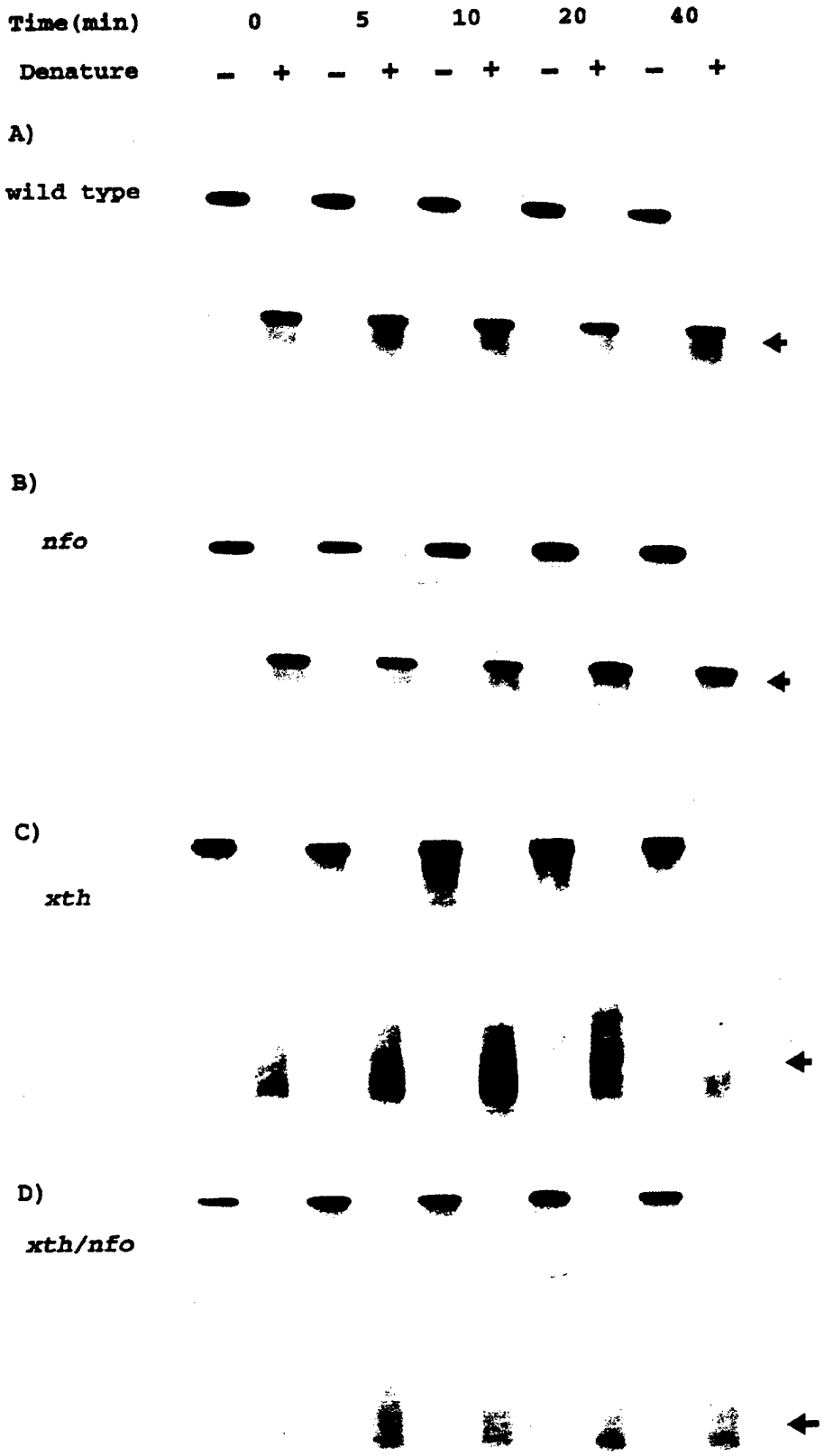
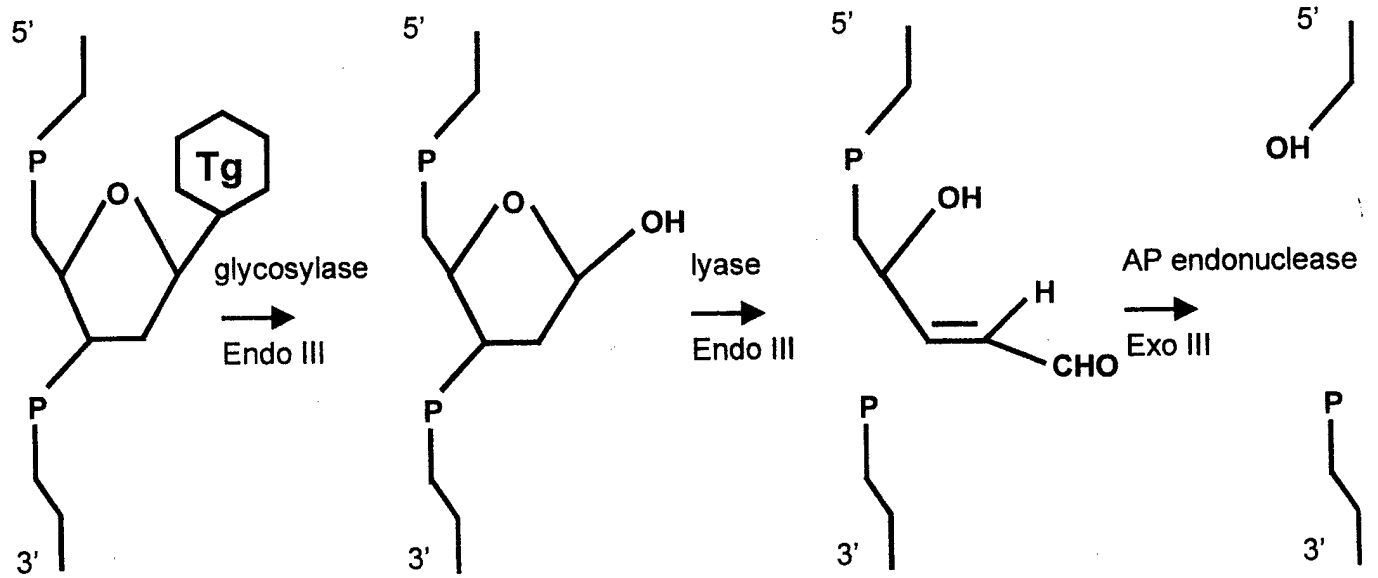


Figure 6



nth mutants:

No Removal of Tg

Cells Survive

xth mutants:

Efficient Removal of Tg
Inefficient removal of abnormal termini

Cells Die

Original Submission June 23, 1997

Original Technical Objectives/Specific Aims - DAMD17-98-1-8201

(1) Determine if TCR of UV-induced damage is deficient in sporadic tumor cell lines, lymphoblastoid and/or tumor cell lines from BRCA1 and BRCA2 patients, fibroblasts or ES cells from mice generated by targeted disruption of the BRCA1 or BRCA2 genes.
(2) Determine if TCR of oxidative damage is altered in the cell lines described above.

The goal of Aim 1 was to determine if we could detect a difference in TCR of UV damage in either human or rodent cell lines with mutations in either BRCA gene. Characterization of such cell lines by other investigators was in its very early stages. Hence, I did not predict the problems that we would have growing such cell lines. So if much of my report sounds technical in nature that is because we had a large number of technical and other problems associated with this and the remaining aims.

We decided not to pursue the mouse ES cell work because by the time the award was made sometime in mid-1998, I knew of work that had already been carried out using mouse ES cells. It was published in 1998. This is the citation: Gowen LC, Avrutskaya AV, Latour AM, Koller BH, Leadon SA. Science. 1998 Aug 14;281(5379):1009-12.

This work covered UV and oxidative damage for BRCA1. It purported that there was no defect in TCR of UV damage but there was a defect in TCR of oxidative damage. I had actually provided the lead investigator of the study with a probe for the mouse DHFR gene that was used in this study (it is cited in the above Science paper). Had I known specifically what the reagent was going to be used for I would not have supplied the reagent because I had proposed similar experiments in this grant application. I had not published anything with the reagent and was under no obligation to share it. However, by the time I realized this, the other laboratory had largely supposedly completed this work. I did not pursue the mouse BRCA2 work because based on seminar presentations at conferences, it appeared that this work was already underway in the Leadon laboratory.

Hence, we changed are attention to human cell lines instead. As I reported in the progress reports, we experienced several problems working with the human cell lines. (1) One problem was in growing sufficient quantities of cells to carry out the TCR assay. This assay requires large quantities of cells. During our attempts to grow them, they would undergo what appeared to growth problems or senescence problems. This was problematic because it is less than ideal to attempt to carry out experiments on what appear to be sick cells. It later became

apparent that the reason for the growth defects is that mutations in these genes create lethality. It is likely that established cell lines with mutations in either BRCA1 or BRCA2 have compensatory mutations elsewhere, otherwise the cell lines would not grow. However, at the time we initiated and continued to carry out these experiments, this was not yet understood. (2) The second problem that we encountered was with our human probe for DHFR. It often times did not give the appropriate sensitivity to quantify data on Southern blots which is what is required to assay either TCR of UV damage or oxidative damage. We tried subcloning and using other methods such as PCR to get sufficient signal. Eventually we did have some success in probes for TCR of UV damage. We used HCC 1937 cells deficient in BRCA1 and CAPAN-1 cells deficient in BRCA2. We saw no defect in TCR of UV damage. (3) The third problem was that when we attempted studies using the antibody to thymine glycol generated by the Leadon laboratory to measure TCR of oxidative damage in BRCA1 or BRCA 2 mutants, we found that in our hands the antibody did not work in an immunoprecipitation reaction. We repeated experiments many times to convince ourselves that we were actually damaging the cells and introducing the appropriate lesions. My background prior to this had not been in oxidative damage, it was with UV damage. Hence, initially I thought the problem was on our end. However, more recently there have been several retractions made by Dr. Leadon or certain journals, most notably a retraction of the 1998 Science paper. I have included the citations to these retractions below and the PNAS "correction". My understanding is that it is likely that more will be forthcoming.

-Science. 2003 Jun 13;300(5626):1657

-DNA Repair (Amst). 2003 Oct 7;2(10):1157

-Cancer Res. 2003 Jul 1;63(13):3846.

-DNA Repair (Amst). 2003 Mar 1;2(3):361

-PNAS | September 30, 2003 | vol. 100 | no. 20 | 11816

Hence, as a stated in my last report, I conclude that we find no evidence for a defect in TCR of UV damage associated with mutations in either BRCA1 or BRCA2. We did, however, see what appeared to be a modest reduction in global repair of UV damage in the BRCA1 mutant. However, given the growth deficiencies associated with mutations in this gene, it is unclear what that means. This has been explored by another investigator.

Nat Genet. 2002 Sep;32(1):180-4. Epub 2002 Aug 26.

I can draw no conclusions regarding a defect in TCR of oxidative damage associated with mutations in BRCA1 or BRCA2. My laboratory was unable to

repeat the assay described for measuring thymine glycol which I published in a 2002 Nucleic Acids Research paper and to my knowledge, no one else has either. Given the content/specifics of the Science retraction and the PNAS correction, there is concern that at least parts of the Leadon work are not credible.

We are currently attempting to use a different antibody, an antibody to bromodeoxyuridine, to measure repair of oxidative damage. We initially did not pursue this for several reasons. One, it lacked specificity and second, based on our experience with the other antibody, I had reservations about the immunological approaches. As stated at the end of the Science retraction, the other lead author who generated the cell lines, is fully willing to now share the cell lines with other investigators to re-examine this potentially important question. It is my opinion that someone does indeed need to repeat the work. However, someone needs to first establish a reproducible and credible assay in order for that work to be re-examined.

(3) Examine the sensitivity of the cell lines described above to UV irradiation or ionizing radiation.

Given that we did not see a defect in TCR in the human cell lines and because of their associated growth problems, I did not see the purpose of attempting to do these experiments.

(4) Examine microsatellite instability in the cell lines described above.

Other groups have done this and it is my impression that there is no clear association.

(5) Determine if transcription on damaged DNA templates is altered in BRCA1 and BRCA2 mutant cell lines.

(6) Attempt to investigate the nature of the genetic defect in any sporadic tumor cell line that we find to be deficient in TCR.

Given our findings of an absence of a defect in TCR of UV damage and my skepticism regarding the oxidative damage data and inability to measure it, neither aim seemed worth pursuing.